

On Phosphatases and Oxido-Reductases in Rat Oral Mucosa

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Abstract Oxido-reductase and phosphatase activities in oral mucosa of rats were analyzed with histochemical and biochemical methods. An even distribution of the oxido-reductase activities were found in the oral mucosa of fetal, developing and adult rats. The enzyme activities increased with increasing age and was high in the adult rat mucosa. Basal cells contained SDH activity but lacked LDH and G6pDH activity. This was interpreted to indicate that basal cells utilize aerobic energy metabolism. As the cells mature and are pushed upwards away from the capillary oxygen supply, the cells convert to an anaerobic metabolism with increasing activity of LDH and G6pDH. For the detection of LDH in tissue sections it was found that the anaerobic incubation technique was the method of choice and that cyanide, amobarbital and menadione frequently used in LDH incubation media to improve the enzyme detection are unsuitable. The phosphatase distribution pattern in the mucosa was different from that of the oxido-reductases. AlkPases and AMPases showed high activity in the developing rat mucosa and a decreasing activity with increasing age. In certain regions with a junction type of mucosa, small areas with AlkPase and AMPase activity remained in the adult rat mucosa. In basal cells of the developing rat mucosa a specific AMPase was found but no AlkPase activity while the spinosum/granulosum cells of junction regions contained a different AMPase isoenzyme and two AlkPase isoenzymes. It was suggested that their activities remained during development as a result of functional adaptation. AcidPase was evenly distributed in the oral mucosa with increasing activity in the granulosum/corneum layers of the older rats. The granulosum/corneum AcidPase is possibly associated with keratinization while the basal cell AcidPase and the occasionally appearing spinosum AcidPase probably fulfill other functions.		
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Sven Sjögren

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ON PHOSPHATASES AND OXIDO-REDUCTASES IN RAT MUCOSA

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On Phosphatases and Oxido-Reductases in Rat Oral Mucosa

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Lund 1984

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To Charlotte and Johan

CONTENTS

	Page
PREFACE	5
INTRODUCTION	6
MATERIAL AND METHODS	12
RESULTS	18
DISCUSSION	22
SUMMARY	28
LITERATURE CITED	30
ACKNOWLEDGEMENTS	42
PAPER I	45
PAPER II	53
PAPER III	61
PAPER IV	87
PAPER V	109
PAPER VI	115

PREFACE

The thesis is based on the following papers which will be referred to in the text by their respective Roman numerals.

- I Sjögren S, Hammarström L, Larsson Å: Enzyme histochemistry of developing rat oral mucosa. J Histochem Cytochem 29:57, 1981
- II Sjögren S: Acid, neutral and alkaline phosphatases of developing rat oral mucosa. J Histochem Cytochem. In press
- III Sjögren S, Ek-Rylander B, Hammarström L: Characterization of alkaline phosphatases of developing rat buccal mucosa. J Histochem Cytochem (submitted for publication)
- IV Sjögren S, Persson B, Hammarström L: ^{65}Zn and alkaline phosphatase in developing rat oral mucosa. J Histochem Cytochem (submitted for publication)
- V Sjögren S: Lactate dehydrogenase in developing rat oral epithelium. J Histochem Cytochem 32:1, 1984
- VI Sjögren S: Lactate dehydrogenase isozymes in developing rat oral mucosa. A comparative study of LDH biochemistry and histochemistry. J Histochem Cytochem (accepted for publication)



INTRODUCTION

A large number of diseases inflict the oral mucosa of man and alters the appearance of the mucosa. Some of the diseases inflict the epithelium, others the connective tissue and some both tissues. Not only diseases of the mucosa but also diseases in other parts of the body alters the appearance of the mucosal lining. The surface appearance of the tongue for instance has since long been used as a diagnostic tool to read the hydration and health status of the body. Waldenström (90) suggested a connection between the macroscopic changes of the mucosal lining and intracellular processes and continued: "if this way of looking at the problem is correct, the state of surface of the tongue will not only be an important indicator of the degree of hydration in the body, but it will also serve as an indispensable mirror of intracellular oxidative processes". The knowledge of the factors and processes which govern this mucosal alteration is limited. During general metabolic disturbances like iron (69) and zinc (85) deficiencies the mucosa alters its appearance. In the case of iron deficiency it leads to disturbances of the vitamin B complex in the body and to atrophy of the tongue papillae and the mucosal epithelium (69).

Detailed knowledge about the structure and function of the mucosa and of the cell metabolism in the different regions of the oral mucosa are necessary not only for the understanding of mucosal physiology and clinical appearance of the mucosa but also for the understanding of how mucosal changes can reflect a state of disease in other and distant parts of the body.

Macroscopically the oral mucosa varies slightly in colour, texture and surface appearance in different parts of the oral cavity. In principle it is a protective membrane barrier and consists of an outer epithelium with a supporting connective tissue. The same organization of

the epithelium and the connective tissue is found in oral mucosa of all mammals (59). However there are species differences in the mucosal appearance. In the rat for instance the entire oral mucosa is keratinized whereas in man only some regions are keratinized, while others are more or less non-keratinized (78,79). Not only the surface keratin layer varies but the whole epithelium shows regional variations in thickness, cristae design, mitotic frequency, cell size and cell content. Also the connective tissue content shows regional variations in collagen fiber composition, in vascular and nerve supply and in mucous gland density (41,78,79).

In man the oral mucosa has classically been divided into different anatomical regions but the oral mucosa may also be classified according to what specific function it is believed to maintain in the various parts of the oral cavity. Functional regions are the mucous secreting regions (sometimes also called the lining mucosa), the masticatory regions and the specialized regions. The mucous secreting regions have a flexible and mobile mucosa which is lined by a more or less non-keratinized squamous epithelium and include soft palate, floor of the mouth, the undersurface of the tongue, the labial and buccal mucosa. The masticatory regions are exposed to load and friction and are lined by a tightly attached mucosa with an orthokeratinized epithelium. They include gingiva and the hard palate. Specialized regions contain sensory organs and taste buds and include the lip mucosa and the dorsal and ventral surfaces of the tongue. (for extensive review the reader is referred to (41,59,79)).

Within a clinically seemingly homogenous region like the buccal mucosa, there are nevertheless small variations in appearance generally believed to express a functional adaptation of the mucous membrane to its environment. It is not known if these alterations are reflected also in the cell metabolism, but it seems reasonable. Several studies have shown that the cell

metabolism varies at least between different parts of the oral cavity although to a small extent (28,77). Many enzymes of mucosal metabolism have been studied biochemically and their role in different types of anabolic or catabolic processes have also been analyzed. The histochemical demonstration of enzymes in tissue sections have for practical reasons been restricted to only a few enzymes, which can be studied with reproducible and reliable techniques. The most studied enzymes have been oxido-reductases of the energy metabolism and non-specific phosphatases and esterases believed to function in the keratinization process. The studies have been concerned mostly with gingiva of humans and laboratory animals. Buccal and palatal mucosa have also been studied although less frequently (for review of the role of enzymes in oral epithelium the reader is referred to Listgarten (50) and Jarrett (41)). In general terms previous studies may be summarized as follows:

Signs of anaerobic metabolism as demonstrated by lactate dehydrogenase (LDH E.C.1.1.1.27.) activity have been found in the epithelium of both humans and laboratory animals. The LDH reaction is most intense in the basal cell region with decreasing intensity towards the surface. LDH activity has also been shown in the connective tissue of the oral mucosa (21,26,29,36,45,46,53,64,65,66,72,73,75,92). No major species divergence from this pattern seems to have been reported.

Glucose-6-phosphate dehydrogenase (G6pDH E.C.1.1.1.49.) activity is found almost exclusively in the spinosum and granulosum cells of gingiva, palate and buccal mucosa. The enzyme staining reaction seems to increase towards the surface. Only low activity has been reported in the basal layer and none in the connective tissue of the oral mucosa (21,31,35,39,55,64,65,72,75,88). Signs of G6pDH activity is considered to indicate activation of alternative glycolysis, the so called pentose shunt and to supply lipid and nucleotide metabolism with precursors

(48). No species divergence from this pattern has been reported.

Succinate dehydrogenase (SDH E.C.1.3.99.1.) activity has been demonstrated in basal cells of the epithelium with decreasing activity towards the surface. The exact level at which SDH ceases to be active varies in different reports. However, differences in staining, tissue preparations and methods are important factors which influence the SDH staining result (21). Most authors though, seem to agree that the enzyme staining reaction is concentrated at the lowest layers of the oral epithelium and at the connective tissue of oral mucosa (21,26,27,39,45,46,53,64,65,66,75,88,92). SDH activity is considered to show an active citric acid cycle and an aerobic metabolism (48). No divergence in staining pattern between different species have been reported.

With phosphatases the situation is somewhat different and research of these enzymes have been more contradictory than research of oxido-reductases has been. Most frequently studied has acid phosphatase (AcidPase E.C.3.1.3.2.) been. Most authors agree that AcidPase activity can be found in all layers of the epithelium, with increasing activity towards the outer layers (4,28,37,41,49,70,71,74,76,77,83,87,92). AcidPase is considered to be a marker for the keratinization process in squamous epithelium. It is of lysosomal origin (19) believed to dissolve plasma membranes and organelles prior to the keratinization process (77).

The existence of alkaline phosphatase (AlkPases E.C.3.1.3.1.) in squamous epithelium has been the subject of controversy over the years, although its presence in the fine capillaries of the connective tissue in oral mucosa is generally agreed upon (26,37,49,54,80,92). However, Staple (80) reported additional AlkPase activity in all epithelium layers except in the basal cells of the inflamed ferret gingiva. Lisanti (49) and Winer et al (92) also found AlkPase activity in the inflamed gingiva

epithelium of humans but only in a few cells of the germinative layers. Itoiz et al. (37) studied AlkPase activity in gingiva epithelium and found traces of activity in the horny layer but only in cat and dog, while man, hamster, guinea pig, mouse and rat lacked AlkPase activity in the epithelium. Flannagan and Porter (26) found no evidence of AlkPase activity in palatal epithelium of humans, neither did Magnusson and Linde (54) in mice. AlkPase is a plasma membrane bound (25) zinc metallo enzyme (68) with unknown functions.

5'nucleotidase (AMPase E.C.3.1.3.5.) activity in oral epithelium have only been analyzed in a few studies and with different results. Itoiz et al. (38) reported AMPase activity in rat gingiva epithelium located at the basal layer and the keratinized layers. Palatal and gingival mucosa in the developing mouse was analyzed by Magnusson and Linde (54) who found AMPase activity in all epithelial layers. In adult rat palatal mucosa Magnusson et al (55) and Klaushofer and Böck (44) found AMPase activity only in the basal and the lower spinosum layers of the epithelium. AMPase is a twin enzyme to AlkPase in many tissues and believed mostly to fulfill catabolic functions in disintegrating cells (33,34).

Adenosine triphosphatase (ATPase E.C.3.6.1.3.) activity, finally, have been found in oral mucosa connective tissue, but no activity was reported in oral epithelium (54,66,94)

It appears from the literature cited above that no single study has analyzed the entire oral cavity for regional variations in activity of these commonly studied enzymes. It also appears that the knowledge of enzyme metabolism and activity in oral mucosa is limited only to a few regions from different species. Some of the enzymes reviewed above have in many other tissues been shown to consist of a subgroup of enzymes - isoenzymes, where the isoenzym pattern in many tissues is specific. No analysis of this phenomenon have been performed on oral mucosa.

PURPOSES

The purpose of the thesis was to map commonly studied enzyme activities of the oral mucosa with reliable and well established methods and to analyze the isoenzyme pattern of some of these enzymes.

In summary the aims of the studies have been:

to describe the regional distribution and developmental variations in the rat oral mucosa of LDH, G6pDH, SDH, AcidPase, AlkPase, AMPase and ATPase activities.

To describe the tissue specific isoenzyme pattern of oral mucosal LDH, AcidPase, AlkPase and AMPase with different histochemical methods and biochemical separation techniques.

As the project progressed it gradually became obvious that the histochemical methods currently used for detection of lactate dehydrogenase were inadequate and the results obtained with different methods were to some extent even contradictory. Hence the studies were expanded with a methodological analysis of LDH histochemistry.

MATERIAL AND METHODS

Animals

Sprague Dawley rats of different ages (fetus, developing (10-day-old), and adult) (I) and developing rats only (II-VI) were analyzed.

Methods for obtaining freeze-dried tissue sections for enzyme histochemistry (I-VI) and for autoradiography (IV).

Freeze-dried tissue sections were obtained with a cryostat technique developed for autoradiographic purposes by Ullberg (84). The animals were anaesthetized with ether, mounted on a microtome stage and frozen to a solid block in hexane cooled with dry ice (-75°C). Sagittal, frontal or horizontal 20 μm sections were cut at different levels of the oral cavity and freeze-dried in the sectioning freeze box. Prior to use they were brought to room temperature in an airtight box in order to avoid condensation of humidity on the sections which destroys the morphology and may cause artefactual result. The sections were used either for enzyme staining or for autoradiography.

Enzyme histochemistry of tissue sections (I-VI)

1. LDH was demonstrated by the incubation of tissue sections in a viscous medium prepared according to the method of Chayen et al. (15). Phenazine methosulphate (PMS 0.2 mg/ml), menadione (0.33 mg/ml), amobarbital (10 mM) and cyanide (1 mM) were added alone or in combinations in order to demonstrate their claimed effects (15,22,52,60,61,62,91) on the LDH staining reaction.

Incubation for LDH was also done in an aqueous medium prepared as described by Barka and Anderson (6) (for details see material and methods I,V, VI).

In the histochemical analysis of LDH activity in oral mucosa (V) the same sections were incubated in presence of 1 mM fluoropyruvate, to inhibit the aerobic type of

LDH (61), 3.25 M urea, to inhibit the anaerobic type of LDH (42,60) and 1 mM HgCl_2 , to inhibit all LDH activity (60).

Histochemical demonstration for LDH activity under strictly anaerobic conditions were also done with a medium prepared according to Chayen et al. (15) (VI).

2. The G6pDH activity was demonstrated by incubating sections in a viscous medium as described by Chayen et al. (15) (I).

3. SDH activity in the cells was demonstrated by incubation of the sections in a medium prepared after Chayen et al. (15) (I).

4. Incubation for NADH diaphorase (E.C.1.66.99.3) (V) was performed according to Chayen et al. (15). NADH diaphorase was also demonstrated with the sections incubated in another medium as described by Lojda et al. (52) (VI).

5. AcidPase activity was demonstrated by incubating tissue sections in a medium at pH 5.0, prepared after Barka and Anderson (6) and Lojda et al. (51). The sodium salts of 100 mM fluoride, 100 mM tartrate and 0.1 mM molybdate and 10 mM copper sulphate were added to the reaction medium in order to discriminate between different isoenzymes of AcidPase (32) (II).

6. Non specific AlkPase was shown in tissue sections (I-IV) with the sections incubated as described by Burstone (14). Incubation was also done with a "Gomori type" of medium after a description of Mayahra et al. (58). This was done in the AlkPase inactivation tests to eliminate the influence of exogenous zinc on the AlkPase reaction which occurs in the "Burstone type" of reaction (II). The coupler salt of the "Burstone medium" contains zinc which may restore the catalytic activity of zinc

deprived inactivated AlkPase (43,93). The sections were incubated at room temperature for 15-30 minutes. The inhibiting or promoting effect of pretreatment with EDTA (II,IV) with formaldehyde and glutaraldehyde (II) and with heat (+56°C) (III) was studied. The inhibiting effect of L-p-bromotetramisole addition (12) (II,IV) was also analyzed.

7. AMPase activity was shown (I,II,IV) by incubating sections in a medium which was prepared according to Wachstein and Meissel (89) and modified by Magnusson et al., (55).

The same procedures and inhibitors as used for AlkPase (see above) were added in order to discriminate between different AMPases and between AMPase and AlkPase activity (II,IV).

8. ATPase activity was shown (I) by incubation of sections according to the method of Wachstein and Meissel, (89).

Control incubation was done regularly with omission of either substrate or the capturing ion in the phosphatase incubations. For dehydrogenases either substrates or the coenzyme was left out from the incubation medium.

Tissue preparations for chromatographic procedures, electrophoresis and all assays of enzyme activities (III,IV,VI).

Ten day-old rats were killed and their buccal mucosa rapidly and carefully dissected free from underlying tissues with a blunt instrument in order to prevent contamination of muscles. The mucosa was homogenized in a buffer solution at +4°C. The amount of, the composition of and the pH of the homogenization buffer solutions varied between the individual studies (for details see

material and methods III, IV,VI). In some studies bone (III) heart, liver and skeletal muscles (VI) were included for reference purposes.

Chromatographic procedures (III)

The dialyzed enzyme solution was added at $+4^{\circ}\text{C}$ to a column (83.3 x 1.6 cm) of Sephacryl S-200 superfine^R. One ml fractions at a flow rate of 78ml/h were collected and assayed for AlkPase activity. The AlkPase containing fractions were pooled, dialyzed overnight against 0.1 M tris-HCl, pH 9.2, and added to an equilibrated column of DEAE⁺-Sephacryl^R. One hundred ml buffer was used to elute non-binding peaks. The peaks bound to the DEAE⁺-Sephacryl were obtained using 400 ml of 0.1 M NaCl in 0.1 M tris-HCl, pH 9.2.

Enzyme assays (III,VI)

1. Crude mucosal extract and fractions obtained after the chromatographic procedures were assayed for AlkPase activity in 0.05 M carbonate/bicarbonate buffer, pH 10.0, containing 0.2 mg/ml p-nitrophenylphosphate (p-NPP) and 10 mM MgCl_2 at 37°C and the Michaelis-Menten constant and the pH optimum determined for the individual fractions. Also fractions preincubated for 10,30,60 and 90 minutes in $+56^{\circ}\text{C}$ were assayed for AlkPase activity (III).

2. Tissue homogenates and supernatants from oral mucosa, skeletal muscles, liver and heart were assayed for LDH activity at pH 8.9. (9,11,56) and monitored at 340 nm and $+37^{\circ}\text{C}$. The inhibitory influence of 0.5 and 1.0 mM cyanide was studied at varying concentrations of lactate and in a 0.05 M tris-HCl buffer, pH 8.9, with 1 mM NAD and 1 international unit of rabbit M_4 LDH/ml added to the medium (VI).

Electrophoretic procedures and enzyme histochemistry of gels (III, IV, VI).

1. The polyacrylamide gel electrophoretic system used for the separation of the AlkPase isozymes (III,IV) was a modified 7.5% acrylamide gel, pH 8.3 (18,24,57). Twenty μ l samples of oral mucosa and bone with sucrose added was layered on top of the gel rods and under the buffer. The electrophoresis was run for the first five minutes with 1 mA/gel and after that with 2 mA/gel at a constant voltage. The electrophoresis was stopped after five hours and the gels incubated for AlkPase at pH 9.2 according to the method of Taswell and Jeffers, (82) and after modifications made by Beck et al. (8). Some gels were preincubated in distilled water at $+56^{\circ}\text{C}$ for 10 or 20 minutes before the incubation for AlkPase activity (III).

2. A different system was used for the separation of LDH isozymes (VI). Ten μ l of supernatant from the oral mucosa, skeletal muscles, liver and heart were layered on top of 5.5% acrylamide gel rods, (18,20), with trisglycine buffer (0.05 M tris and 0.38 M glycine), pH 8.3, as electrode buffer and the electrophoresis was run for 45 minutes and with 2.5 mA/gel. Gels were incubated for LDH at pH 7.4 (20) (VI).

To the LDH medium was also added either 10 mM amobarbital or 1 mM cyanide. Incubations were also done where PMS of the medium was exchanged for menadione (0.33 mg/ml). Some gels were incubated for NADH diaphorase (E.C.1.6.99.3.) at pH 7.4 (52) (VI).

Control incubations were made by omitting either substrate or coenzyme.

Autoradiography and radioactivity assay (IV)

Eight 4-day-old littermates were given repeated intraperitoneal injections of ^{65}Zn and freeze-dried tissue

sections in three different planes were taken from three of them. The sections were pressed against Structurix X-ray film and left for one week's exposure, after which the plates were developed. The oral mucosa from the remaining five rats were dissected out and the supernatant of the mucosal homogenate was separated on acrylamide gels (18,24,57). Some gels were divided in 2 mm slices and placed in special plastic tubes and assayed for radioactivity from ^{65}Zn . Other gels were incubated for AlkPase (8,82) (IV).

RESULTS

General observations

All the enzymes investigated (I-VI) could be demonstrated in the rat oral mucosa. However, the activity and the distribution of the individual enzymes varied from region to region and between different layers of the mucosa. Conspicuous and intense AlkPase and AMPase activities were observed in specific areas, such as the buccal fold and the junctions between hard and soft palate and between attached gingiva and alveolar mucosa. The buccal fold is a sometimes sessile and sometimes fold-like structure, which extends from the upper incisor region via the commissural area to the lower incisor region. Distally it extends only for a short distance (I; figures 1a-c). Its major portion is situated at the commissural area and it varies in size between different animals. In the 10-day-old rat it is prominent but as the animals mature the fold diminishes in size. In the adult rat there is no fold left. Instead a thin line of hair follicles and sebaceous glands is found in this region. In the border zones between phosphatase active and non-active areas of the developing rats, the cells with activity were mixed with cells without activity in an apparently randomly fashion.

Significant age related differences were found for AMPase and AlkPase and with increasing age areas with activity of the two enzymes were found to decrease in size. AcidPase and oxido-reductases showed a general activity increase with development.

Individual enzymes

1. LDH activity of rat oral mucosa studied with a viscous medium technique (I,V,VI) and anaerobic incubation conditions showed that rat oral mucosa contained LDH activity mostly in the epithelium and concentrated to the spinosum granulosum cells with weak or no activity seen

in the basal layer. The connective tissue contained negligible activity of LDH. No regional or age related variations were seen. The methods for demonstrating LDH in tissue sections were shown to be insufficient and even give incorrect information about LDH localization (V). This was shown to depend on addition of cyanide and menadione to the incubation medium (VI). Cyanide addition caused competitive inhibition of LDH activity by complex binding to the NAD molecule. Menadione addition inhibited LDH activity and caused a tissue staining which was indistinguishable from that caused by NADH diaphorase. It was concentrated at the basal layers of the oral epithelium. This type of "LDH staining" corresponded with LDH staining obtained with an aqueous incubation medium (V). Finally it was shown that oral mucosa contained five LDH isoenzymes mainly anaerobic and that the zymogram showed great similarities with that of skeletal muscle LDH (VI).

2. G6pDH activity caused intense staining in oral mucosa epithelium. However, basal cells were almost devoid of activity and the connective tissue was completely blank. No age related or any regional differences of significance were observed (I).

3. SDH activity was found in all basal cells with no particular regional differentiation and no age related differences. All other mucosal layers more or less lacked signs of activity of this enzyme (I).

4. Acid phosphatase activity was intense in the upper epithelial layers at all ages. A moderate staining was also seen in the basal cell layer. Only a weak AcidPase activity could be seen in the spinosum cells and in the connective tissue (I,II). When the analysis of AcidPase activity of different localization in the developing rat oral mucosa was expanded with application of inhibition

tests they seemingly discriminated between the AcidPases of different localization (II). Thus four AcidPases were found with different characteristics and localization: one in the connective tissue cells, a second in the basal cells, and a third in the granulosum/corneum cells, all three found in the whole of the mucous membrane. The fourth a molybdate sensitive AcidPase of low activity was found only in the buccal fold spinosum cells (I,II).

5. Alkaline phosphatase activity showed regional as well as age dependant variations in the oral mucous membrane. In the fetus AlkPase activity was seen in the whole oral mucosa epithelium except basal cells. With increasing age the number of regions with epithelium AlkPase activity decreased and the activity was confined only to junction type of mucosa. The AlkPase activity of these sites were localized at the connective tissue capillaries and the spinosum/granulosum cells of the epithelium (I). One of these regions was the buccal fold of the developing rat oral mucosa where the AlkPase was shown to consist of three different isoenzymes with two localized at the epithelium and the third at the capillaries (II,III, IV). All three showed genuine AlkPase characteristics such as substrate and pH optima (III) and a sensitivity to L-p-bromotetramisole (II,IV). They reacted differently to heat inactivation (III) with capillary AlkPase being most sensitive while the two epithelial were quite resistant to heat inactivation. All three AlkPases were shown to be inactivated by prolonged EDTA treatment (II,IV). The activity could be restored with zinc addition but not with magnesium. The distribution of mucosal AlkPase showed a remarkable resemblance with the uptake pattern of injected radioactive zinc in the oral epithelium of developing rats (IV). The mucosal AlkPases were shown to be zinc dependant with inhibition and reactivation tests but mucosal extract from ^{65}Zn injected rats applied to electrophoretic separation showed that

the AlkPase isoenzymes and the retained ^{65}Zn migrated separately during the electrophoresis (IV).

6. Regional and age dependant distribution of AMPases in the oral mucosa showed great similarities with that of AlkPases with a few exceptions. In addition to the epithelial spinosum/granulosum activity basal cell AMPase activity was found in most regions of the oral mucosa. AMPase activity was also found in capillaries of the oral mucosa (I). The basal cell AMPase activity was completely inhibited by prolonged EDTA treatment while the spinosum granulosum AMPase retained weak activity after this treatment (II). All activity which was inhibited with EDTA could be restored by reactivation with zinc but not with magnesium (IV). The AMPase activity of oral mucosa was resistant to formaldehyde and L-p-bromotetramisole, but sensitive to glutaraldehyde and EDTA (II).

7. Activity of ATPase was found only in the connective tissue layers of the mucosal layers and stained intensely even after relatively short incubation times. No age depending differences of importance were registered and only a very slight variation in enzyme activity was registered between different regions. Mobile regions and tongue papillae were slightly more stained from ATPase activity. No epithelial ATPase activity was seen (I).

DISCUSSION

It is evident from the compiled results (I-VI) that even small and seemingly homogenous areas of the rat oral mucosa show a rich variation in enzyme activities. This was particularly true for the developing animals but also in the adult rat regional differences in enzyme activities were registered (I). The lack of consistency with which AMPase and AlkPase in particular appeared in the oral mucosa may partly explain why the presence of these enzymes have caused so much confusion in the literature over the years (26,37,38,44,49,54,55,80,92). In the adult rat the activities appeared so scarcely that only systematic serial sectioning or the mere chance would reveal their existence. This random appearance may suggest that the activities have remained during the development of the oral mucosa possibly as a consequence of functional adaptation.

Among the variations in localization of enzymes the greatest variation was found for the phosphatase group while the oxido-reductases showed only minor variations. SDH was the only one which consistently had the same high activity in the basal layer of the whole epithelium, a finding in accordance with earlier studies (21,26,27,39,45,46,53,64,65,66,75,88,92). Presence of SDH activity indicate that basal cells of squamous epithelium in general utilize aerobic metabolism (48).

In the spinosum/granulosum layers the enzyme pattern differed from the one in the basal layer. As the epithelial lining cross over from skin to oral mucosa a drastic increase in the activity of G6pDH was found (I) in the spinosum/granulosum layers. The findings are in agreement with those of others (41) and may be interpreted to show that although skin and mucosa both are covered by a keratinizing squamous epithelium their cells have a different type of lipid and nucleotide metabolism. Basal cells seemingly differ from spinosum/granulosum cells in their energy metabolism. For instance SDH

activity decreases abruptly in the cells as they mature into spinosum cells and move away from the oxygen supply of connective tissue capillaries. Instead an anaerobic type of energy metabolism prevails with increased LDH activity.

The lack of evidence of LDH activity in basal cells (I, V,VI) was a surprising result considering previous reports (21,26,29,36,45,46,53,64,65,66,72,73,75,92). This lack of activity was suspected to be artefactual and depend on differences in incubation methods (I). In previous reports without exceptions, water based incubation media have been used. In such media more than 90% of the LDH enzyme is lost from the tissue section to the medium within minutes (1). It may be concluded that this type of media demonstrates NADH diaphorase activity rather than LDH activity (I,V,VI).The observation may also be true for other tissues than the mucosa but this aspect was not pursued.

Some of the additives like cyanide, amobarbital and menadione used in the incubation media to improve the tissue staining reaction had an inhibiting effect on the LDH activity and cannot be recommended to be used for histochemical demonstration of LDH (I,V,VI). Particularly the inhibitory effect of cyanide was unexpected although the complex binding ability have been known for a long time (16,95). The property of cyanide to bind NAD must have implications not only for the histochemical LDH reaction but also for all those other enymes where the two compounds are mixed in the incubation medium.

When the more complicated anaerobic incubation technique was used it was found that oral epithelium contains mainly anaerobic LDH in the spinosum/granulosum cells. The basal cells contain very little if any LDH activity whereas they are rich in their content of NADH diaphorase. Thus the current concept of LDH beeing very active

in basal cells with decreasing activity towards the surface has to be reevaluated.

In the osteoclasts, lactate generated by LDH is believed to remove calcium phosphate from mineralized tissues. This activity of lactate to remove calcium have been proposed by Jarrett (41) to be another aspect of how epithelial cells may use LDH. Calcium is considered to be an important factor for the stabilization of cell membranes and for the maintenance of cell attachment. Thus apart from utilizing LDH to regenerate NAD it produces lactate which may help the cells to dissociate in the desquamation process. Also the labilizing effect on membranes from a low pH value in the tissues may assist in the desquamation process and in the dissolvment of organelles in the keratinization process. Such a low pH may be produced in the cell by an increased production of lactate which occur as a consequence of anaerobic metabolism and activation of LDH, particularly in the granulose cells.

Another divalent cation which is important for the stabilization of membranes and for supporting the cell-cell attachment is zinc (4,29,71). It is required for the maintenance of a normal appearance of oral mucosa and skin (2,3,4,29,63,67,71,85). Deficiency leads to an increased parakeratosis and an increased activity of LDH and AcidPase in squamous epithelial cells (4,29,71). Injected radioactive zinc is accumulated in the oral epithelium (7). The accumulation of zinc in the oral epithelium (IV) was not uniform along the epithelial lining but rather concentrated at specific regions some of which were also rich in their content of AlkPases and AMPases (I,II, III and IV). Zinc as an integrated and active part in a number of enzyme systems like LDH, alcohol dehydrogenase, AlkPase etc (13,68) may have accumulated in the mucosa as part of such enzymes.

However, the AlkPases identified by different techniques (II,III and IV) and shown to exist in squamous

epithelium of the buccal fold of developing rats were also shown to be zinc dependant with characteristics of zinc-metallo enzymes (II and IV). Although the retention pattern of zinc in oral epithelium was similar to but not identical with the one seen for AlkPase it was not possible to show that the accumulated zinc was build into the AlkPase molecule. In fact the results suggested that the zinc was loosely attached to unknown substances of the oral mucosa cells since it dissociated during the electrophoresis. Zinc metallo proteins are stable and remain intact during normal purification procedures while zinc-protein aggregates may dissociate during e.g. electrophoresis (68,86).

If the retention of zinc in oral epithelium does not reflect conjugation with any enzyme, zinc may still be required as a membrane stabilizing agent (4,29,71). Thus in areas of the oral mucosa where a greater demand to withstand stretching forces are required of the cells there must also be a requirement of factors helpful in this respect. Zinc may be one of them.

AMPase and AlkPase are often considered to be twin enzymes with identical localization (30). In oral mucosa this was true in a sense when looking at the spinosum/granulosum activities (I,II,IV). However the basal cell AMPase had no AlkPase counterpart and was shown to be of a different type (II). Whether its function is also different from that of spinosum/granulosum cell AMPase is not known but is conceivable. The selective appearance of AlkPases, AMPases and a specific molybdate resistant AcidPase in the buccal fold epithelium (I-IV) suggests that they are induced by or maintained by the same unknown local factor(s) and it may also suggest that their functions are related. AlkPase activity may be induced and released from biliary ducts after ligation (10). The phenomenon of induced AlkPase activity have been extensively studied in tail skin epithelium of rats by Jarrett and associates (40) and their studies conclude

that vitamin A acetate and applied pressure both induce activity in squamous epithelium of several phosphatases with an alkaline pH optimum. Jarrett (41) suggested functions of cell growth regulation for these phosphatases. AMPase (5) as well as AcidPase (23) are known to be concentrated to areas in lower biological systems where cellmembrane growth is required. These suggested functions are plausible also for the special phosphatase activities of buccal fold epithelium.

In paper I AcidPase activity was found in the connective tissue and in the epithelium. The epithelial distribution with activity in the basal cells, markedly decreased activity in spinosum cells and again an intense activity in the granulosum/corneum cells raised the suspicion of an inhibiting factor of AcidPase intervening in the spinosum layer. By their different characteristics found with inhibition tests (II) it was concluded that although this is possible they probably represented different AcidPase isozymes possibly with different functions.

Jarrett (41) suggested that basal cell AcidPase dissolve spindle bodies that are formed during the mitosis while spinosum AcidPase activity digest desmosomes after exocytosis of the enzyme into the extracellular space. Both functions are conceivable for the mucosal AcidPases of the same localization.

The AcidPase found in granulosum/corneum cells was intensely active and the degree of activity increased with age (I). A possible explanation for this may be that the degree of keratinization in the oral epithelium increases as the rats develop and mature. This AcidPase is believed to participate in the keratinization by dissolving organelles and membranes (41,47). A more specified and direct role in the keratinization biochemistry have been forwarded by Dale (17) who has isolated a phosphate containing precursor (philagrin or basic protein) which upon removal of the phosphate aggregates to form keratin.

Phosphate hydrolyzing enzymes like AcidPase may thus be considered to be plausible candidates for a direct role in the keratinization process.

SUMMARY

Oxido-reductase and phosphatase activities in oral mucosa of rats were analyzed with histochemical and biochemical methods.

An even distribution of the oxido-reductase activities were found in the oral mucosa of fetal, developing and adult rats. The enzyme activity increased with increasing age and was high in the adult rat mucosa. No significant regional differences were found, whereas there were clear differences between the individual mucosal layers. Basal cells contained SDH activity but lacked more or less LDH and G6pDH activity. This was interpreted to indicate that basal cells with their proximity to connective tissue capillaries utilize aerobic energy metabolism. As the cells mature and are pushed upwards away from the capillary oxygen supply, the cells convert to an anaerobic metabolism as shown by an increaseing activity of both anaerobic LDH and G6pDH activity. It was suggested that LDH and G6pDH in the outer layers provides the cell with energy. However they may also serve other functions which could assist the cell in the keratinization and desquamation process. For the detection of LDH in freeze-dried tissue sections it was found that the anaerobic incubation technique was the method of choice and that substances like cyanide, menadione and amobarbital frequently used in LDH incubation media to improve the enzyme detection are unsuitable and should be avoided.

The phosphatase distribution pattern in the mucosa was different from that of the oxido-reductases. AlkPases and AMPases showed high activity in the developing rat mucosa and a decreasing activity with increasing age. However in certain regions with a junction type of mucosa, small areas with AlkPase and AMPase activity remained in the adult rat mucosa. There were also activity differences between the individual layers. In basal cells of the developing rat mucosa a specific AMPase was found but no

AlkPase activity while the spinosum/granulosum cells of junction regions contained a different AMPase isoenzyme and two AlkPase isoenzymes. The functions of the AlkPases and AMPases are unknown but since they appeared selectively at specific sites it is tempting to suggest that their functions are connected with specific qualities of these regions, perhaps as a result of functional adaptation.

AcidPase was more evenly distributed in the oral mucosa with increasing activity in the granulosum/corneum layers of the older rats. This was suggested to be connected with the increasing degree of keratinization occurring with increasing age. The granulosum/corneum AcidPase is possibly associated with the keratinization while the basal cell AcidPase and the occasionally appearing spinosum AcidPase probably fulfill other functions.

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Enzyme Histochemistry of Developing Rat Oral Mucosa

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The oral mucosa of developing and mature rats was analyzed histochemically for regional enzyme differences. The following enzymes were studied: nonspecific alkaline phosphatase (alkpase), acid phosphatase (acidpase), 5'-nucleotidase (AMPase), adenosine triphosphatase (AT-Pase), succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), and glucose-6-phosphate dehydrogenase (G-6-pDH). All enzymes were active in the oral mucosa, but regional as well as tissue variations were observed.

Epithelium in all regions showed acidpase staining. Oxidoreductases were found in all regions with variations within the epithelium. The epithelium of specific regions stained for alkpase and AMPase, while adjacent epithelium did not. We suggest that the alkpase and AMPase activities are associated with specific functions of the epithelium in these regions.

KEY WORDS: Enzymes; Epithelium; Oral mucosa; Rats.

Introduction

It is well-known that the different regions of the human oral mucosa show variations in histologic structure and degree of keratinization (for review see Dolby (1975)). These morphologic variations may be the result of a functional adaptation, mainly due to stress during mastication or they may be due to some evolutionary process. The variations may also be of significance in the development of disease. Many oral diseases involve the epithelial lining, e.g., those associated with abnormal keratinization. Knowledge of the properties of the epithelium in the different regions of the oral cavity is therefore important for it increases our understanding of regional variation in the development of disease as well.

Little is known about any significant metabolic variations in the different parts of the oral mucosa. A number of studies have however appeared on the presence of different enzymes in the oral mucosa. Most frequently studied are oxidoreductases (Löe and Nuki, 1966; Gerson, 1967; Flanagan and Porter, 1971; Heyden and Verhaug, 1971; Heyden, 1974; Nuki et al., 1976), acid phosphohydrolase (Itoiz et al., 1964; Squier and Waterhouse, 1967; Flanagan and Porter, 1971; Gerson, 1973; Larmas, 1976), 5-nucleotidase (Hiatt et al., 1974; Klaushofer and Böck, 1974; Magnusson and Linde, 1974; Magnusson et al., 1974), and nonspecific esterases (Itoiz et al., 1964; Squier and Waterhouse, 1970; Winer et al., 1970). Activity of alkaline phosphatase in oral epithelium has been reported (Staple, 1957; Itoiz et al., 1964; Winer et al., 1970), but others have failed to demonstrate such activity (Flanagan and Porter, 1971). These studies have been carried out on different species and on different parts of the oral mucosa. To our knowledge, no efforts have been made to

screen the whole cavity in order to locate regional activity differences.

The purpose of the present study was to examine all areas of the oral mucosa of the rat of different ages with histochemical techniques for the presence of enzyme activities, particularly in the epithelium. The sectioning techniques that we used permitted us to study the whole of each individual plane of the cavity. The enzymes were selected on the basis of previously reported results in the literature (see above), including enzymes involved in energy production as well as enzymes proposed to function in transport, catabolic, and anabolic processes. The present studies are intended to form the basis of future experimental studies on the development of pathologic processes in the rat oral mucosa.

Material and Methods

Male and female Sprague-Dawley rats of different ages were used. In all, fetuses removed from the uterus on approximately the 19th day of gestation, 10-day-old, and 6-month-old rats were studied. All rats were anesthetized with ether and instantly frozen (-75°C) on a microtome stage in carboxymethylcellulose dispersed in water. Sagittal, frontal, and horizontal sections $20\text{ }\mu\text{m}$ thick were cut at $200\text{ }\mu\text{m}$ intervals and the sections were stabilized by applying adhesive tape (686, Minnesota Mining and Manufacturing Co) on the cutting surface. The whole procedure was performed in a freezebox (-20°C), where the sections were left to dry. The method has been described in detail by Ullberg (1954). In serial sections the different enzymes were demonstrated by incubation in the media described below. The oral mucosa was defined as the connective tissue of lamina propria and the covering squamous epithelium of the oral cavity extending from the hairy part of the lips to the pharynx.

Chemicals. All chemicals used were of analytical grade and obtained from Sigma Chemical Co. St. Louis, MO. PVA (grade G04/140) was purchased from Cytochemical Co. Edware, Middlesex.

Lactate dehydrogenase ("LDH" E.C. 1.1.1.27). LDH was prepared by dissolving 50 mM sodium lactate in 0.05 M glycyl-glycine buffer, pH 8.0, with 5 mM nitroblue tetrazolium (NBT) and 3 mM NAD (nicotinamide adenine dinucleotide) (Chayen et al., 1973). To optimize incubation conditions 22% polyvinyl alcohol (PVA) and 0.2 mg phenazinmethosulfate (PMS)/ml were added to the incubation medium, which was saturated with nitrogen before use. Incubation was carried out at 37°C for 15 min.

Succinate dehydrogenase ("SDH" E.C. 1.3.99.1). SDH was prepared by dissolving 50 mM sodium succinate in 0.05 M glycyl-glycine buffer, pH 8.0, with 0.4% NBT, 22% PVA, and 0.2 mg PMS/ml (Chayen et al., 1973). This solution was saturated with nitrogen before use. Sections were incubated in 37°C for 30 min.

Glucose-6-phosphate dehydrogenase ("G6pDH" E.C. 1.1.1.49). To prepare G6pDH, 5 mM NBT was dissolved in 0.05

mM glycyl-glycine buffer, pH 8.0, and 22% PVA, 3 mM nicotinamide adenine dinucleotide (NADP), and 5 mM glucose-6-phosphate were added. Just before use 0.2 mg PMS/ml was added and the solution was saturated with nitrogen. Sections were incubated in 37°C for 5 min.

Acid phosphatase ("acidpase" E.C. 3.1.3.2). Acidpase was a product of combining 0.5 mg/ml of α -naphthyl acid phosphate as substrate and 0.15 ml hexazotized pararosaniline/ml reaction medium in 0.2 M acetate buffer, pH 5, for 30 min at 37°C (Barka and Anderson, 1962; Lojda et al., 1964).

Nonspecific alkaline phosphatase ("alkpase" E.C. 3.1.3.1). Alkpase was produced by combining 0.09 mM naphthol-As-Mx-phosphate as substrate and Red Violet LB or Fast Blue RR as coupler in 0.2 M Tris HCl buffer, pH 8.3 (Burstone, 1958). Sections were incubated at room temperature for 30 min.

Adenosine monophosphatase (AMPase E.C. 3.1.3.5) and adenosine triphosphatase (ATPase E.C. 3.6.1.3.) The AMPase medium (Wachstein and Meisel, 1957) was modified according to

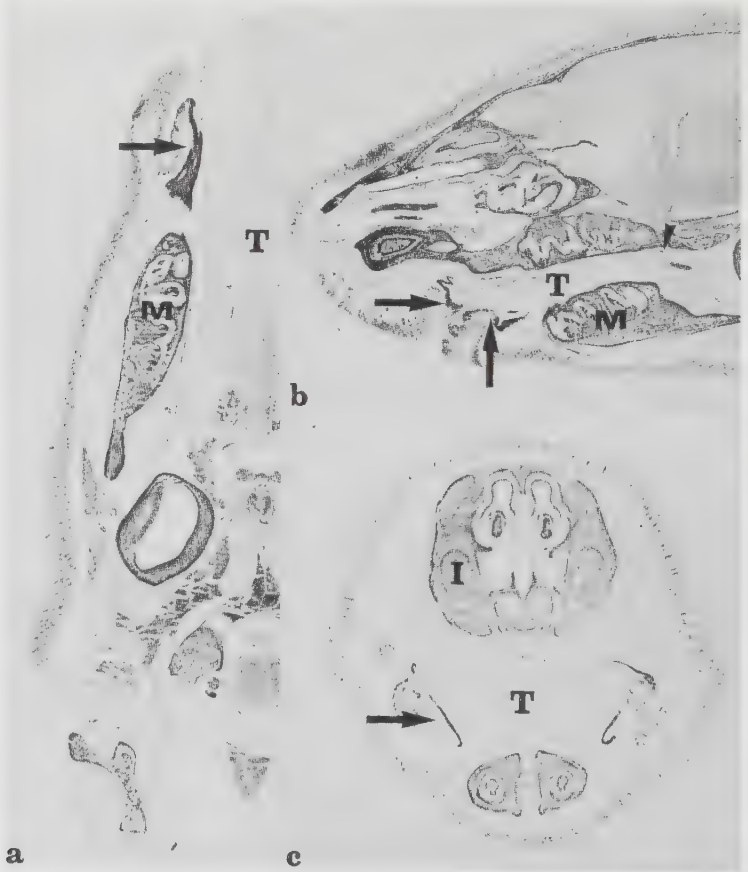


Figure 1. Sections from 10-day-old rat stained for alkpase, pH 8.3. (a) Horizontal section; (b) sagittal section; (c) frontal section. Arrows indicate buccal fold with alkpase activity. Arrowhead indicates activity at junction between hard and soft palate. Note also alkpase activity in hard tissues. T = tongue, M = molars, I = incisor. $\times 4$.

Magnusson et al. (1974) using 3.6 mM adenosine 5-monophosphoric acid (AMP) and 3.4 mM lead nitrate with 10 mM $MgCl_2$ in 80 mM Tris-maleate buffer, pH 7.2. The ATPase medium (Wachstein and Meisel, 1957) consisted of 2 mM adenosine triphosphoric acid (ATP), 2 mM lead nitrate, and 1 mM $MgCl_2$ in 80 mM Tris-maleate buffer, pH 7.2. Incubations were carried out for 15 min at 37°C.

Lead phosphate was visualized by ammonium sulfide diluted to 10% in 0.9% ice-cold NaCl. All sections were mounted in glycerin jelly.

Control incubations in all media were performed either without substrate or after preincubation in 65°C for 10 min.

The incubated sections were studied in a stereo or light microscope. The degree of staining was considered to reflect the degree of enzyme activity in those regions where controls showed no staining. Staining observed in oxidoreductase enzyme control sections was, however, attributed to "nothing dehydrogenase" (Zimmermann and Pearse, 1959). Any variation of staining intensity was interpreted with great care, since it was not possible to standardize and control section thickness.

Results

General Observations

Activity could be demonstrated in the rat oral mucosa of all the enzymes investigated. However, the activity and the distribution of the individual enzymes differed between different regions, between different layers of the mucosa, and between rats of different ages. Significant enzyme activities were observed in certain areas, among these were the buccal fold (a circumscribed, fold-like structure inside the orifice of the mouth, lateral to the incisors) and the junction between hard and soft palate. We called these "junction areas," since they seem to represent the borders between different types of mucosa (Figure 1a-c). A more detailed discussion of these observations follows.

Individual Enzyme Patterns

Oxidoreductases. LDH and G6pDH activities were found at all ages and in all layers of the epithelium. The highest activities were generally found in the upper spinous layers of the epithelium, where G6pDH show strong and LDH intermediate activity. SDH activity appeared mainly in the basal layers of the epithelium. In the connective tissue low staining of SDH and LDH was found, while G6pDH showed almost no staining (Figure 2a-c).

Acidpase. Acidpase activity (Figures 3d, 4f) was intense in the upper epithelial layers at all ages. A moderate staining was also seen in the basal cell layers. Only a low acidpase activity could be seen in the connective tissue.

Alkpase. In the fetal rats all epithelial layers except basal cells showed a definite staining for alkpase. With increasing age the areas with activity gradually diminished and in the older rats activity was seen only in the junction areas of the mucosa (Figure 4). Activity was observed in the connective tissue at the tongue papillae and in parts of the lining mucosa, particularly in the small blood vessels.

AMPase. In the epithelium almost all basal cells at all ages showed a definite AMPase staining, but for the other epithelial layers, age and regional differences were observed. In the fetal rats, all epithelial layers of the entire mucosa showed staining, but this staining was confined in the older animals to the junction areas (Figures 4b, 3b). Activity was seen in the connective tissue at the dorsum of the tongue and also in association with a large number of small blood vessels.

ATPase. ATPase activity was found in the connective tissue, but not in the epithelium. Slightly more stained areas were observed in the papillae of the tongue and also in mobile mucosa (Figure 3c).

Tissue Distribution of Enzymes

Connective tissue. The lamina propria stained weakly for all enzymes except ATPase, which stained rather intensely in most parts of the mucosa (Figure 3c). Alkpase activity was recored in the subepithelial blood vessels (Figure 4e).

Epithelium.

Basal cell layer. Almost all areas of the mucosa showed a definite basal cell staining for AMPase at all ages. The basal cells stained for acidpase (Figure 4d) and SDH (Figure 2a) in growing rats, while other oxidoreductase activities were only randomly seen. Little or no alkpase staining could be detected in the basal cells (Figure 4a).

Spinous cell layer. This layer stained for alkpase and AMPase in the fetal mucosa (Figure 4a, b). In older rats corresponding activity was confined to the junction areas. All ages and regions except the buccal fold showed a very light staining for acid pase and definitive staining for oxidoreductases. SDH, however, showed lower activity than in the basal cells.

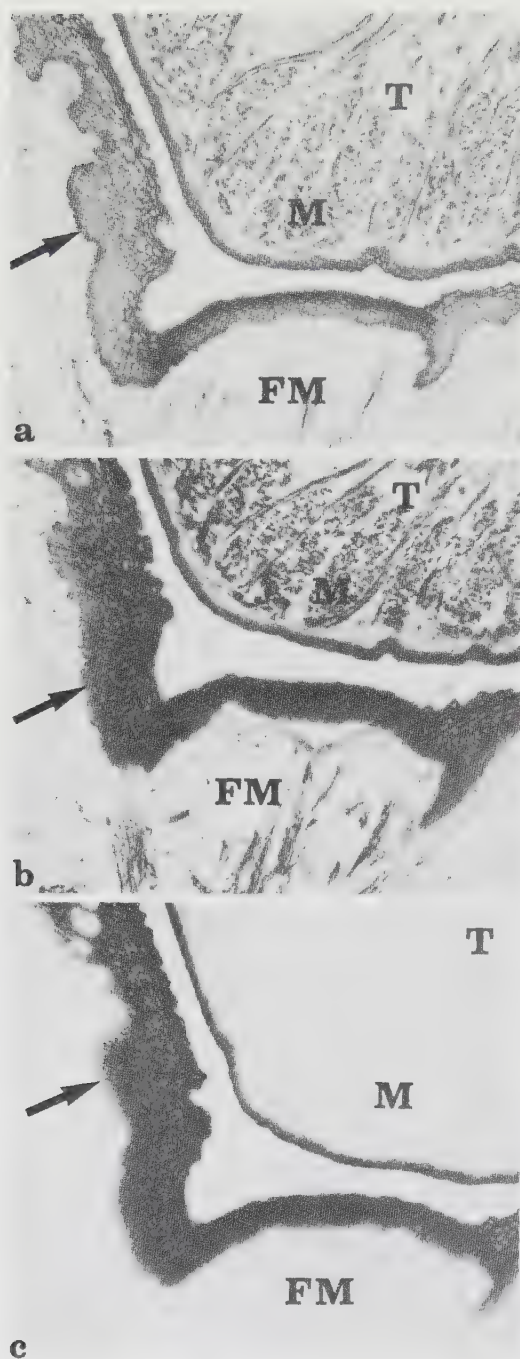
Outer cell layers. These layers stained intensely for acidpase in all regions of the mucosa and at all ages (Figure 3d). With respect to the other enzymes, the granulous layer showed activities similar to those of the spinous layer.

Age Related Distribution of Enzymes

Significant age related differences were found for AMPase and alkpase. With increasing age the number of cells with the two activities seemed to decrease in the epithelium. In the oldest rats, activity was seen only in the junction areas (Figure 4a, c, e).

Regional Distribution of Enzymes

A unique distribution pattern for alkpase and AMPase was found in the epithelium. In the older rats, activity of these enzymes was confined to the junction areas. Here, AMPase activity was more widespread than alkpase, the latter appearing more randomly, and in the buccal fold mainly in the posterior portion of the fold (Figure 3a, b). Specialized



mucosa of the tongue also showed staining of alkase and AMPase in both the connective tissue and the epithelium. The connective tissue of the junction and tongue regions seemed to stain more intensely for ATPase than in other areas, but this regional difference was not very distinct.

Another portion of the oral mucosa with a different staining pattern was the junction between oral and reduced enamel epithelium. The connective tissue of this area stained particularly intensely for alkase and the basal cells stained for AMPase. In the upper spinous layer AMPase as well as acidase and oxidoreductases, particularly G-6-pDH, were observed. Alkase could not be demonstrated in this epithelium.

The epithelium of the circumvallate papilla of the tongue stained intensely for AMPase and alkase. This staining was localized to the crypt of the papilla, seemingly concentrating in the upper third of the epithelium (Figure 5a). In fixed, paraffin-embedded routine stained sections, this area was found to be rich in taste buds.

The sectioning technique used made possible observations of enzyme activity in other regions of the experimental animals as well. We observed that the mucosal epithelium of the anal and vaginal openings stained for AMPase and alkase. The staining was confined to the outer cell layers (Figure 5b).

Discussion

The enzymes studied in this article have previously been analyzed in human oral mucosa as well as in the mucosa of other species including rats. In these studies most of the oxidoreductases and phosphatases could be demonstrated in all mucosal cell layers. The enzymes were particularly active in the epithelial cells (reviewed in Listgarten (1972)), and our findings are in accordance with these results.

The histochemical demonstration of enzyme changes in oral mucosal tissues undergoing structural changes with increasing age has not been previously reported. In the present study, the development of the different parts of the oral mucosa was accompanied by a reduction of the number of cells exhibiting alkase and AMPase activity, suggesting a possible correlation between these enzymes and embryonal remnants. AMPase has, however, also been associated with the catabolism of nucleotides in disintegrating mature cells (Hardonk 1968; Hardonk and Koudstaal, 1968). In the outer layers of the epithelium, such a function for the enzyme may seem logical, but the additional AMPase activity observed in the basal cells raises the question whether there are two different functions for AMPase within the epithelium. The

Figure 2. Detail of frontal section of 10-day-old rat. Showing oxidoreductase activity in lower part of buccal fold, cf. Figure 1c. (a) Staining of SDH is seen in basal cells (arrow) and in muscle cells (M). The staining in the upper epithelial cell layers was also seen in controls ("nothing dehydrogenase"). (b) Staining of LDH is concentrated in the upper layers of the epithelium and is only sparsely present in the basal cells (arrow) as compared to controls. Muscle cells stain intensely. (c) Staining of G-6-pDH is concentrated mainly in epithelial cells of the mucosa. Staining increases gradually from basal cells (arrow) towards surface cells. FM = floor of mouth, T = tongue, M = cross-sectioned striated muscle cells. $\times 110$.

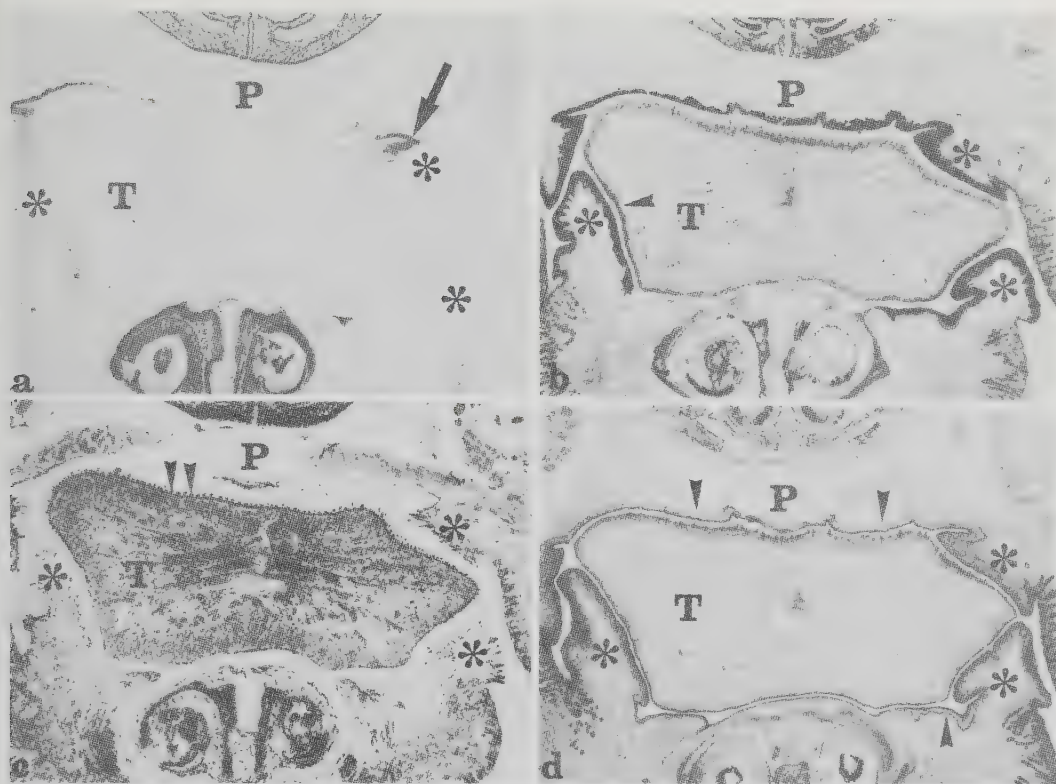


Figure 3. Frontal sections of 10-day-old rat, showing the distribution of phosphatases. (a) Alkpase staining is almost exclusively seen in relation to the buccal fold epithelium (arrow). The staining is less distinct and more arbitrarily localized than the corresponding AMPase staining of Figure 3b. (b) AMPase staining is seen in all layers of the epithelium of the buccal fold and of the lateral border of the tongue (arrow head). (c) ATPase staining is particularly intense in the tongue muscles (T) and in the lamina propria of tongue papillae (arrowheads) and of the buccal fold (asterisks). (d) Acidpase staining is seen in the outer epithelium and in the basal epithelium cell layer of the entire mucosa (arrowheads). The spinous layer is less stained except in the buccal fold where a moderate staining is seen. Asterisk = buccal fold, P = palate, T = tongue. $\times 15$.

basal cell activity may be associated with anabolic functions in cellular growth, e.g., plasma membrane synthesis (cf. Panda et al. (1962), El-Aaser and Reid (1969), Hobbs et al. (1969)).

We found alkpase activity in those structures where AMPase seemed to be the most active and widely spread of the two enzymes, which may suggest only one enzyme active on the different substrates. However, in all basal cells, where AMPase activity was found alkpase activity was lacking. Our results indicate that two or more phosphatases with a neutral or alkaline pH optimum may be present within localized regions of the rat oral mucosa.

Previously reported alkpase activity in keratinizing epithelium has been much debated. Staple (1957), Itoiz et al. (1964), and Winer et al. (1970) all reported enzyme activity in keratinizing epithelium, while others failed to demonstrate the enzyme's presence (Flanagan and Porter, 1971). These conflicting results may well reflect differences in the selection of biopsy material. The results obtained by the whole body sectioning technique used in the present study clearly demonstrated that alkpase activity in adult animals is restricted to localized regions of the mucosa, requiring careful selection of biopsy site for its demonstration.

Alkpase has usually not been associated with kernatinizing epithelium, nevertheless it has been suggested that the enzyme may function in the process of "high pressure keratin" production (Jarret, 1973). The oral epithelium of the rat is orthokeratinized in all regions (Squier and Meyer, 1971). Therefore, our findings of alkpase activity appearing only in some parts of the mucosa indicate that this enzyme is not directly involved in the keratinization process in the rat oral mucosa.

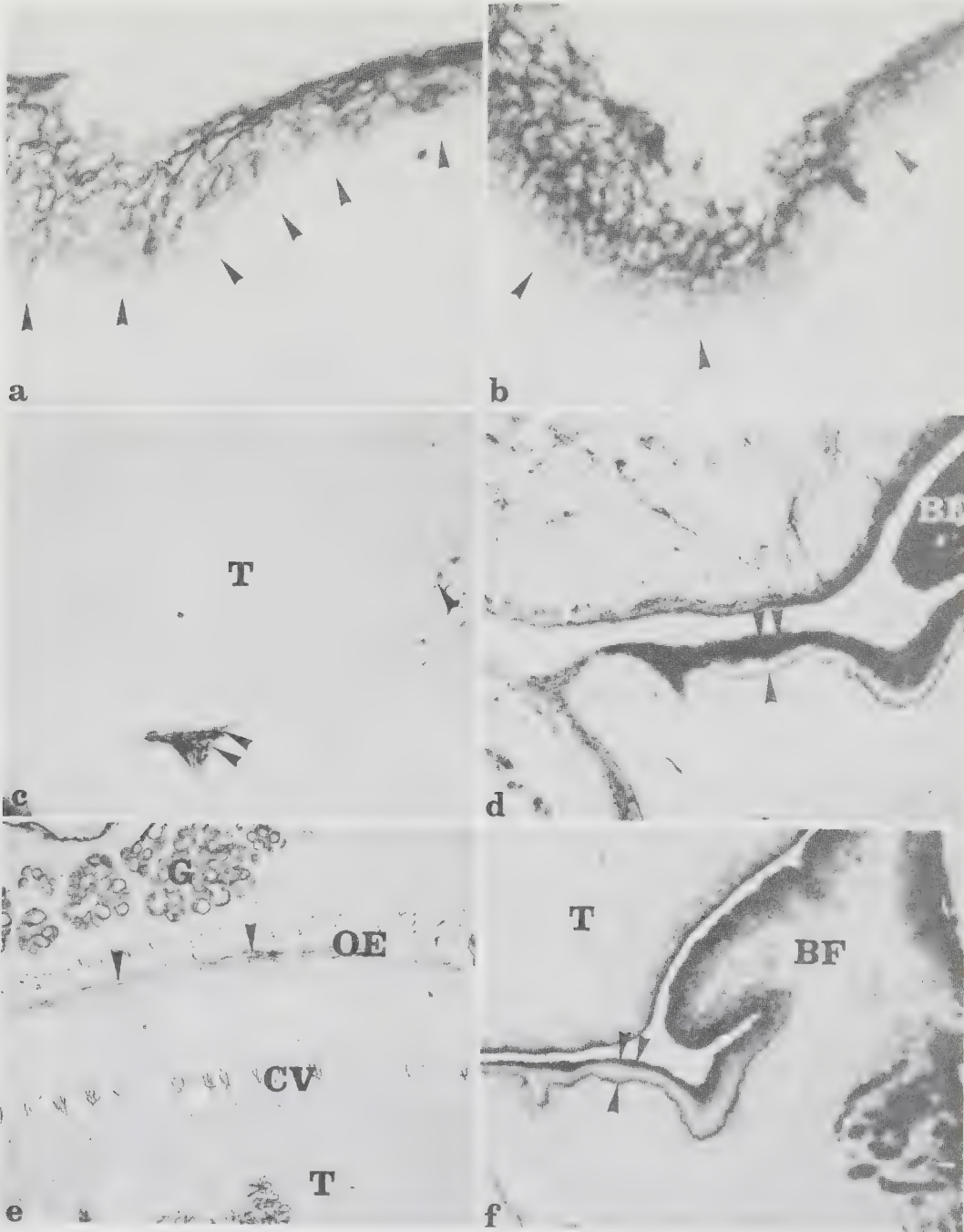


Figure 4

Figure 4. Details of acidpase, alkpase, and AMPase distribution. (a) Detail of buccal fold from sagittal section of rat fetus. Alkpase, pH 8.3. Arrowheads indicate localization of nonreactive basal cell layer. $\times 225$. (b) Same area as (a) stained for AMPase pH 7.2. $\times 225$. (c) Detail of frontal section from 10-day-old rat stained for alkpase, pH 8.3. There is an abrupt transition (double arrow heads) of stained epithelium to unstained epithelium of the floor of the mouth. Single arrowhead points to stained blood vessels. T = tongue. $\times 40$. (d) Same area as (c) stained for AMPase, pH 7.2. Note the stratified pattern with staining of basal cells (arrowhead) and outer layers (double arrowheads), while lower spinosum layer is unstained. Compare with epithelium of buccal fold (BF), where the staining is found in all layers. T = tongue. $\times 40$. (e) Detail of frontal section from junctional area of adult rate stained for alkpase. Note the randomly appearing cells in upper spinosum-granulosum-corneum layers which stain for alkpase (arrowheads). OE = oral epithelium of soft palate, G = mucous glands, CV = capillary vessels, T = tongue. $\times 40$. (f) Same area as (c) and (d) stained for acidpase, pH 5.0. Note basal cell staining (arrowhead) and corneum layer (double arrowheads) staining. Compare with staining of entire epithelium of the buccal fold (BF) with somewhat less staining in basal layer. T = tongue. $\times 40$.

Another enzyme proposed to be intimately associated with the keratinization process is acidpase (reviewed by Jarret (1973)). We found enzyme activity in the epithelium of the entire mucosa, which is in agreement with previous results (Listgarten, 1972). The finding of acidpase staining in the basal and the granulosum layers, with reduced staining in the spinous layer, is suggestive of inhibition of the enzyme in the spinosum layer.

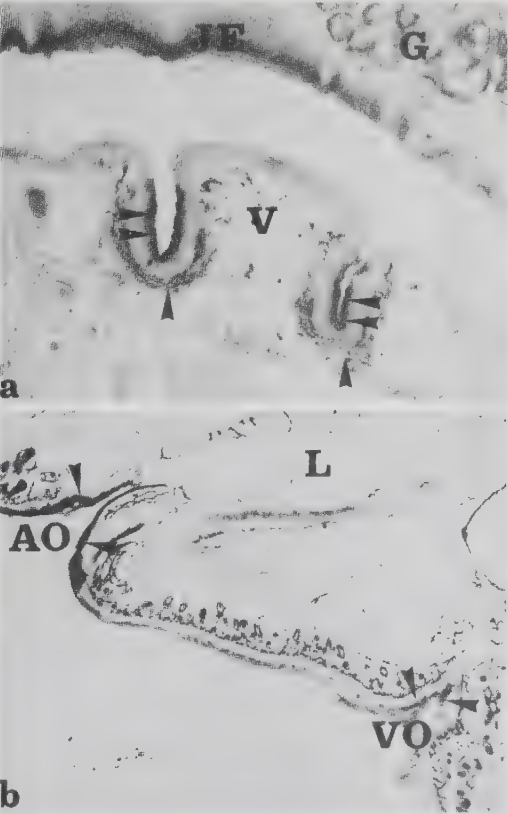
We found no regional differences in oxidoreductase activity. Like Heyden and Verhaug (1971) and Jarret (1973), we observed a low staining of G6pDH in the basal cell layer, with an increased staining in the upper spinous and granulosum layers. This staining pattern was interpreted by Jarret (1973) to indicate an anaerobic glycolysis in the upper epithelial cells. In the basal cells of the rat oral mucosa, aerobic glycolysis (SDH staining) seems to function as the primary source of energy. In this respect there seems to be no obvious species differences between rat mucosa and human mucosa.

However, we also noted a slight increase in LDH staining in the layers above the basal cell layer. This finding is in contrast to that of several previous histochemical (Mori et al., 1962; L  e and Nuki, 1966; Lange and Kirschmann, 1967) and ultramicrochemical (Gerson et al., 1966) studies. We refer in this context to the discussion on dehydrogenase histochemistry in Pearse (1972) and Chayen et al. (1973). From these, it may be concluded that activity of diaphorase as well as diffusion of the soluble LDH enzyme in the tissue may seriously interfere with the histochemical localization of LDH. Some of these undesirable effects may be overcome by adding PVA and PMS to the incubation medium. Our results may thus be related in part to our choice of histochemical method, and we propose to consider the oxidoreductase activity of the rat oral epithelium in greater detail in a separate study.

To summarize our findings, specific enzyme patterns appeared in specific regions of the rat oral mucosa. This specificity was characterized by the activity of alkpase/AMPase in the epithelium of junctional zones. Such regional specificity was not observed with any of the other enzymes investigated, i.e.,

ATPase, acidpase, and the oxidoreductases. It therefore seems logical to assume that the epithelial cells of the regions with alkpase/AMPase activities have functions additional to or different from the cells of the other mucosal areas. Our findings show that this activity is not associated with the keratinization process. We also found alkpase activity in the epithelium at the anal and vaginal openings, and it is tempting to speculate that this enzyme is somehow related to the functional ability of these tissues to stretch. Similarly, the epithelium of the junctional zones in the rat oral mucosa may be functionally adapted to movements of these tissues.

Figure 5. Details from sections of 10-day-old rat stained for alkpase. (a) Demonstrates enzyme staining in the outer third of the epithelium (double arrowheads) of the vallate papilla (V). Staining is seen in the underlying connective tissue (arrowhead) and in the junctional epithelium of palate (JE). The mucous glands of the palate also stained for alkpase. $\times 110$. (b) Sagittal section of anal area of 10-day-old rat showing alkpase staining confined to the outer third of the epithelium (arrowhead) at the anal orifice (AO) and vaginal orifice (VO), L = lumen of lower intestine. $\times 25$.



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Acid, Neutral, and Alkaline Phosphatases in Developing Rat Oral Mucosa

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Developing rat oral mucosa contains high activities of acid (AcidPase), neutral (adenosine monophosphatase AMPase), and alkaline (AlkPase) phosphatases. This study is concerned with a detailed analysis of the distribution of these enzymes in freeze-dried sections of oral mucosa. The sections were incubated for AcidPase, AMPase, and AlkPase in the presence of certain inhibiting agents. Four AcidPases, three AMPases, and two AlkPases were identified in the oral mucosa. Six of them

were found in the epithelium and three in the connective tissue. Three of the epithelial phosphatases were unique in the sense that they were found only in junction mucosa epithelium. The function of these enzymes may be related to the maintenance of the epithelial cell attachment in an area subjected to stretching, possibly by affecting the periphery of the cells.

KEY WORDS: Acid phosphatase; Alkaline phosphatase; Epithelium; Inhibitors; 5'-nucleotidase; Rats.

Introduction

The oral mucosa of humans and other mammals show regional variation in morphology and metabolic activity as indicated by mitotic index, epithelium thickness, degree of keratinization, etc. The variation may reflect a functional adaptation to mechanical stress and friction during mastication (6). Other metabolic parameters have also been studied, e.g., enzymes such as oxido-reductases and phosphatases. Oxido-reductases show different degrees of activity in the epithelial layers of oral mucosa (22,27) and of skin (19). In young and adult rats (34) only minor differences exist between different oral regions. Phosphatases are generally found along the entire mucous membrane, but they also exhibit different degrees of activity in the various layers. However, some phosphatases are selectively localized at specific transitional or junction areas between different kinds of mucosa (34). Such a junction area is the buccal fold, a circumscribed foldlike structure inside the orifice of the mouth and lateral to the incisors (Figures 1a, 2a). All told the phosphatase activities in the mucosa are associated with at least three enzymes: one acid, one neutral, and one alkaline, of which the acid is found at three different levels (34). Previously, Jarrett (19) described acid phosphatase (AcidPase) activity at three levels in squamous epithelium of the skin, but it was not known at the time if the enzyme activity represented one, two, or three different AcidPases.

Alkaline phosphatase (AlkPase) (8) and 5'-nucleotidase (adenosine 5'-monophosphatase AMPase) (10) exist as isoenzymes in various tissues and react differently to activators and inhibitors. Their biological role continues to be debated.

The purpose of this article was to analyze the phosphatase activities in developing rat oral mucosa in general and the buccal fold region in particular, because it is anatomically easy to identify and easily accessible for studies of experimental pathology in oral mucosa. Conventional histochemistry with inhibiting ions was used to discriminate the different phosphatase isoenzymes.

Materials and Methods

Littermates of 10-day-old Sprague–Dawley rats were anesthetized in ether, mounted on a microtome stage in carboxy methyl cellulose dispersed in water and frozen in hexane cooled to -75° with dry ice. Horizontal and sagittal, freeze-dried sections were obtained through a technique developed by Ullberg (35). Serial sections were either untreated or pretreated in 4% formaldehyde for 30 min at $+20^{\circ}\text{C}$ or in 2.5% glutaraldehyde for 30 min at $+20^{\circ}\text{C}$ (16) or treated in 30 mM ethylenediamine tetraacetic acid (EDTA) for 48 hr at $+4^{\circ}\text{C}$ (25,31). Some sections were both fixed and EDTA treated. For AcidPase isoenzyme demonstration only sections pretreated in 4% formaldehyde for 30 min (13) or untreated sections were used. Pretreated and untreated sections were then incubated in the different media described below.

5'-nucleotidase (AMPase) (E.C.3.1.3.5). 3.6 mM adenosine monophosphate (AMP) and 3.4 mM lead nitrate were dissolved in 80 mM Tris–maleate buffer, pH 7.2, with 10 mM magnesium chloride added (29,36). 0.1 mM L-p-bromotetramisole was added to the incubation medium to inhibit nonspecific AlkPase and 0.1 mM D-p-bromotetramisole was added as control (3). After 15 min incubation at 37°C , the section was immersed in ice cold 0.9% NaCl and carefully washed, developed for 10 sec in 10% ammonium sulfide diluted to

1% with 0.9% NaCl, washed in 0.9% NaCl, and mounted in glycerin jelly. Incubation was also performed at pH 10.0 using lead citrate and Tris-HCl buffer instead of lead nitrate and Tris-maleate buffer, pH 7.2. All other incubation conditions remained the same.

Nonspecific alkaline phosphatase (E.C.3.1.3.1). AlkPase was investigated at pH 8.3 according to the method described by Burstone (4) with 0.09 mM Naphthol-As-Mx-phosphate as substrate and Red Violet LB or Fast Blue RR as coupling salt. Incubation was also done with a Gomori medium where 10 mM β -glycerophosphate was dissolved in 80 mM Tris-HCl buffer pH 10.0 to which was added 3.9 mM magnesium chloride and 2.0 mM lead citrate (30). All sections were incubated at 37°C for 15 min, washed in 0.9% NaCl, and developed as described above. Overlapping activity between AlkPase and AMPase was controlled by addition of L- β -bromotetramisole, a potent and selective inhibitor of AlkPase (3).

Acid phosphatase (E.C.3.1.3.2). AcidPase was demonstrated by the method of Barka and Anderson (1), as modified by Lajda et al. (28) and Hammarström et al. (12), using 1 mg/ml of α -naphthyl-acid-phosphate as substrate with 0.01 ml of hexazonized pararosaniline/ml of reaction medium in 0.2 M acetate buffer pH 5.0 for 30 min at 37°C. Sections were also incubated with 1 mM sodium molybdate, 100 mM disodium tartrate, 100 mM sodium fluoride, or 10 mM copper sulfate (12) present in the incubation medium.

Controls. These incubations were performed by omitting substrate in the initial medium and in the Gomori type medium either by omitting the substrate or the capturing ions. All chemicals were purchased from Sigma Chemical Co. except for L- and D- β -bromotetramisole, which were purchased from Aldrich Europe.

Results

General Observations

In the oral cavity AMPase and AlkPase showed similar localizations and were specifically found in the buccal fold (Figure 1 a-d), soft palate, and lateral parts of the tongue. These areas will henceforth be referred to as junction mucosa, since they all are located at the junction between different kinds of mucosa. Staining for AcidPase was found along the entire mucosa (Figure 2a). Differences in phosphatase staining were seen not only between different regions of the oral cavity but also between individual mucosal layers.

Figure 1(a-d) Horizontal sections of the head of 10-day-old rats stained for AMPase and AlkPase. Original magnification $\times 4$. Bars = 2.5 mm. (a) AMPase at pH 7.2 with details from the oral cavity. In particular note staining of the posterior parts (arrowhead), of the buccal fold (asterisks), and of the mucosal lining of tongue (arrows). (b) Same as in a, stained for AMPase at pH 10.0. Note lack of staining along the mucosal lining of tongue (arrows) and compare with the staining seen in a. T, tongue; M, molar of mandible. (c) Same as in a, stained for AlkPase at pH 8.3 with naphthol technique. Intense staining can be seen in posterior parts (arrowhead) of the buccal fold (asterisk). (d) Same as in a, stained for AlkPase at pH 10.0 with Gomori technique, T, tongue; M, mandibular molar. Both AMPase and AlkPase staining occurs in the same region at the buccal fold. Note, however, that the shape of the buccal fold varies between the individual rats and also to some extent with the section level.



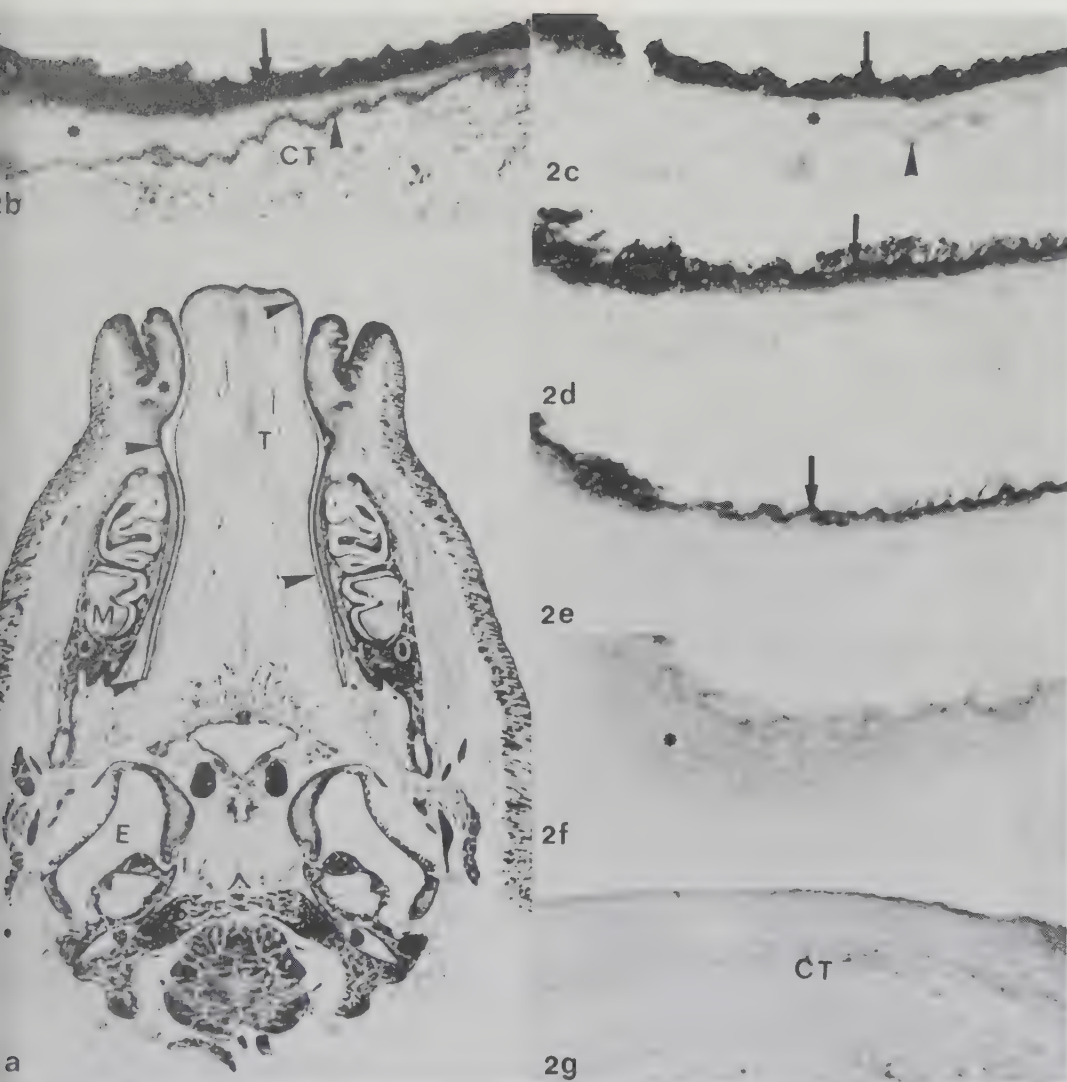


Figure 2(a-g) Horizontal section of head (a) and details of oral mucosa (b-g) from 10-day-old rats stained for AcidPase. (a) Full view of head. No pretreatment and no inhibitor in incubation medium. Note staining along the entire mucosal lining (arrowheads). Asterisk indicates anterior buccal fold. T, tongue; M, mandibular molar; E, inner ear. Original magnification $\times 4$. Bar = 2.5 mm. (b) Detail of posterior part of buccal fold. No pretreatment and no inhibitors in incubation medium. Note staining in connective tissue of lamina propria (CT), basal layer (arrowhead), and, in particular, granulosum/corneum layers (arrow). A slight staining reaction is also seen in the spinosum layer (asterisk). Original magnification $\times 120$. Bar = 100 μm . (c) Detail of posterior part of buccal fold from section pretreated in formaldehyde. The staining reaction is reduced but stain-

ing is still seen in basal layer (arrowhead) and granulosum/corneum layers (arrow). A slight spinosum staining (asterisk) is also seen. Original magnification $\times 120$. Bar = 100 μm . (d) Section stained in the presence of 10 mM copper. The staining is markedly reduced and seen primarily only in the granulosum/corneum layers (arrow). Original magnification $\times 120$. Bar = 100 μm . (e) Section stained with 100 mM fluoride in the medium. Staining reaction is seen only in the granulosum layer (arrow). Original magnification $\times 120$. Bar = 100 μm . (f) Section stained with 1 mM molybdate. Light staining is seen in the spinosum layer (asterisk). Original magnification $\times 120$. Bar = 100 μm . (g) Section stained with 100 mM tartrate. Note discrete staining reaction in connective tissue of lamina propria (CT). Original magnification $\times 120$. Bar = 100 μm .

Tissue Distribution

All basal cells of the epithelium stained for AcidPase and slightly irregularly for AMPase at pH 7.2 as well (Figure 3b). However, they did not stain for either AMPase pH 10.0, or for AlkPase (Figure 3a).

The spinosum cells in oral mucosa were without phosphatase staining in most regions. A few stained intensely, e.g., in junction mucosa epithelium where heavy AMPase and AlkPase staining was seen at all pH levels (Figure 3a,b).

The granulosum/corneum layers along the entire mucosa stained for AcidPase. In junction mucosa AMPase and AlkPase staining was also seen at all pH levels.

In the connective tissue, AcidPase staining was found along the whole mucous membrane. In the tongue and buccal fold

mucosa fine capillaries were stained for AMPase and AlkPase at all pH levels studied.

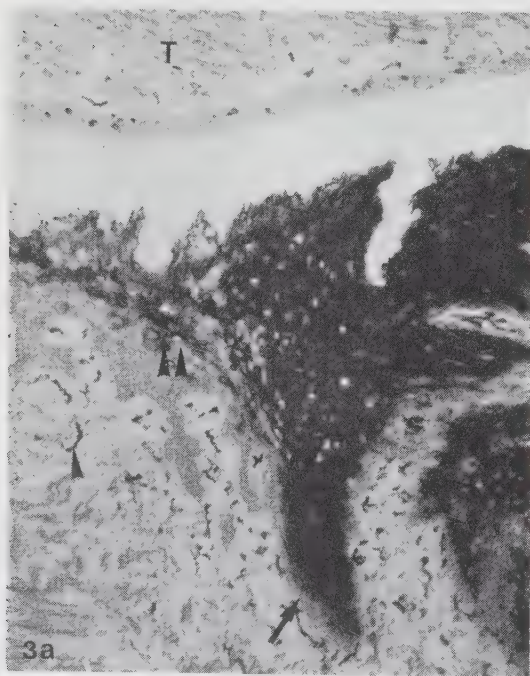
Individual Enzymes

AMPase, pH 7.2. The staining for AMPase, found in basal cells along the entire mucosa and in spinosum and granulosum cells of junction mucosa epithelium, was resistant to fixation in formaldehyde, while glutaraldehyde inhibited all staining. Furthermore, AMPase staining was unaffected by L-p-bromotetramisole, but inhibited by EDTA. There was one exception. After 48 hr EDTA treatment, a weak staining remained in the spinosum cells of junction mucosa epithelium (see Table 1).

AMPase, pH 10.0. This AMPase staining, mainly of spinosum/granulosum cells of junction mucosa epithelium, was enhanced and improved by fixation in formaldehyde and slightly inhibited by glutaraldehyde and L-p-bromotetramisole. It was almost completely inhibited by EDTA treatment (see Table 1).

AlkPase, pH 8.3. This staining method of Burstone (4) was found in most cells of junction mucosa epithelium except basal cells (see Figure 3a). It was inhibited by L-p-bromotetramisole, slightly inhibited by glutaraldehyde fixation, and

Figure 3(a) Section of buccal fold from 10-day-old rat stained for AlkPase, pH 8.3. Note lack of staining in basal layer (arrow) and of random staining in part of the spinosum layer (double arrowheads). Also note staining of fine capillaries in connective tissue (arrowhead). Counterstained with Kernechtrot. Original magnification $\times 120$. Bar = 100 μ m. (b) Section of buccal fold from 10-day-old rat stained for AMPase, pH 7.2. Note staining of basal cells (arrowheads), capillaries (small arrow), and of the adjacent tongue mucosal lining (arrows). The random staining of spinosum cells (double arrows) is less prominent than that seen in a. T, tongue. Original magnification $\times 60$. Bar = 50 μ m.



PHOSPHATASES IN RAT ORAL MUCOSA

Table 1. AMPase and AlkPase staining^a

Inhibitor	5'-Nucleotidase (AMPase), pH 7.2		5'-Nucleotidase (AMPase), pH 10.0		AlkPase, pH 8.3 ^b	AlkPase, pH 10.0 ^c
	Basal cells of entire mucosa	Epithelium of junction mucosa	Basal cells of entire mucosa	Epithelium of junction mucosa	Epithelium of junction mucosa except basal cells	Epithelium of junction mucosa except basal cells
—	++	+++	0	+++	+++	+++
Formaldehyde 4%	++	+++	0	+++	+++	+++
Glutaraldehyde 2.5%	0	0	0	++	++	++
L-p-Bromotetramisole 0.1 mM	++	+++	0	+(+)	0	0
30 mM EDTA for 48 hr at + 4°C	0	(+)	0	(+)	+++	0

^aSemiquantitative evaluation of AMPase and AlkPase staining in buccal fold epithelium and in basal cells of the entire epithelium of 10-day-old rats (see Figures 1 and 3). The epithelium was analyzed for staining at different pH's with or without inhibiting agents. +++ = strong staining; + = low staining; ++ = moderate staining; 0 = no detectable staining.
^bNaphthol-diazonium medium (Burstone)
^cGlycerophosphate-lead medium (Gomori)

slightly enhanced by formaldehyde fixation. Staining was unaffected by 48 hr pretreatment in 30 mM EDTA (see Table 1).

AlkPase, pH 10.0. Incubation for AlkPase in the Gomori type medium at pH 10.0 resulted in a staining pattern similar to the one seen at pH 8.3. However, differences in staining properties were noticed after EDTA pretreatment, whence complete inhibition of AlkPase staining occurred. Fixation or addition of L-p-bromotetramisole affected the enzyme staining in a similar fashion as seen at pH 8.3 (see Table 1a).

AcidPase, pH 5.0. When adding inhibitors to the incubation medium a differentiated staining pattern appeared. Along the whole mucosa, one specific tartrate resistant AcidPase was seen in the connective tissue cells. Another copper-, fluoride-, and formaldehyde-resistant AcPase was seen in the outer layers of the epithelial lining. Basal cell AcPase was inhibited by all the agents used, except copper ions and formaldehyde fix-

ation. Finally, in spinosum cells of buccal fold epithelium a weak molybdate-resistant AcPase staining was seen (Figure 2f and Table 2), while the remaining epithelial cells were unstained in the presence of molybdate.

All controls were negative.

Discussion

This study demonstrated several phosphatases in rat oral mucosa with different characteristics or localization. The staining in capillaries of AlkPase and AMPase and the staining in the epithelium of AcidPase is in accordance with previous reports (10,19). The connective tissue AcidPase was tartrate resistant with low staining intensity and most likely an AcidPase different from that found in the epithelium. Their biological functions may nevertheless be similar or even the same. In general, AcidPase is considered to be a lysosomal enzyme (5) and is believed to dissolve organelles and cytoplasmic content prior to keratinization of cells in the granulous layer (22,26).

Table 2. AcidPase staining. pH 5.0^a

Inhibitor	Connective tissue layer	Basal cells	Spinosum cells	Granulosum/ corneum cells
—	+	++	++	+++
Formaldehyde 4%	0	+	(+)	+++
Sodium fluoride 100 mM	0	0	0	++
Sodium tartrate 100 mM	+	0	0	0
Copper sulfate 10 mM	0	(+)	0	++
Sodium molybdate 1 mM	0	0	+ ^b	0

^aSemiquantitative evaluation of AcidPase staining in oral mucosa from 10-day-old rats. Sections were incubated for AcidPase at pH 5.0 with α-naphthylphosphate as substrate. The evaluation refers to the staining pattern in the entire oral mucosa. +++ = strong staining; + = low staining; ++ = moderate staining; 0 = no detectable staining.
^bNote that molybdate-resistant staining was seen only in buccal fold epithelium. (See also Figure 2a–g.)

However, in lesions like psoriasis and lichen planus, a decrease of AcidPase activity is seen in parallel with an increasing thickness of the keratin layer (20). Furthermore, application of vitamin A alcohol on keratinizing epithelium is known to destabilize lysosomes and release acid hydrolases, including AcidPase, and yet vitamin A caused decreases in keratin thickness and a mucous metaplasia of the epithelium (2). It seems from the literature that the role for AcidPase in the keratinization process has yet to be fully elucidated and requires further study.

The enzyme has also been found in the spinosum layer where it is believed to digest desmosomes after being exocytosed into the extracellular space. A third localization for AcidPase in squamous epithelium is in the basal layer where it is believed to remove spindle bodies formed during mitosis (for review of AcidPase in keratinizing epithelium see ref. 19).

The results in the present investigation show AcidPase staining in three different locations of the epithelium: one occurring over the entire basal layer, a second only in the spinosum/granulosum layer of junction mucosa, and a third in the granulosum/corneum layers of the entire mucosa. The AcidPases of the different locations may possibly have separate functions, such as those suggested for epidermal AcidPase by Jarrett (19). However, a general cell function have been proposed that involves lysosomal AcidPase for sublethal modification of cell periphery and hence a release of cell detachment (9,33,37-39), thus enabling cells to partly release themselves from surrounding structures and permit increased mobility without cell destruction. Such an ability seems advantageous for cells in stretched tissues such as junction mucosa and its presence should therefore be considered.

Incubation of untreated sections for AlkPase at pH 8.3 or 10.0 resulted in the same staining patterns, but with clear differences after EDTA treatment. Either there are two AlkPases in oral epithelium having different sensitivity to EDTA or, what is more likely, the two methods for demonstrating AlkPase at pH 8.3 and 10.0, respectively, affect the AlkPase staining differently after EDTA treatment. The diazonium salts used in the Burstone medium at pH 8.3, but not in the Gomori medium at pH 10.0, are rich in zinc (23) and thus reactivate EDTA-inactivated AlkPase during incubation (25,40).

Staining from AlkPase, pH 8.3, has previously been demonstrated in the granulosum layer of human dermal squamous epithelium (18) and shown to degrade lipids, such as lecithin, in the keratinization process (19). This function may also be valid for AlkPase in oral epithelium of the rat. If so, however, one would expect the AlkPase staining to be more consistently present and not appear so randomly in junction mucosa epithelium (see Figure 3). Thus other functions proposed for AlkPase should also be considered, e.g., transport of molecules over membranes (8), or membrane degradation (11), or influence over epithelial cell proliferation (21).

The observations made with AMPase staining indicate the presence of at least two enzymes in oral epithelium of different sensitivity to EDTA. One AMPase appears in basal cells and the other in the spinosum/granulosum/corneum cells. The findings of sensitivity to glutaraldehyde (16) and resistance to L-p-bromotetramisole (3) are consistent with true AMPase ac-

tivity. The fact that some staining was resistant to glutaraldehyde at pH 10.0 may indicate that there are three AMPases, of which two are found in spinosum/granulosum/corneum cells. More likely, however, this staining represents glutaraldehyde-resistant AlkPase interfering at this high pH level. Such a conclusion was supported by the stain reducing effects of L-p-bromotetramisole at this pH level.

AMPase is generally believed to function in the catabolism of nuclear material in disintegrating cells (14,15), a function quite feasible in spinosum/granulosum/corneum layers, but less so in basal cells, where the possibilities of other functions such as plasma membrane synthesis in cell proliferation (7,17,32) or transport of nucleosides into the basal cells (24) must also be considered.

In conclusion, AcPase in rat oral epithelium seemed generally to be most active in the keratinizing layers of the entire epithelium and also, but to a lesser extent, in basal cells. AMPase activity was generally found in basal cells and in connective tissue capillaries together with AlkPase. However, specific and apparently unique AcidPase, AlkPase and AMPase activities were localized at a junction area of the oral mucosa—the buccal fold. The seemingly random activity among the epithelial cells (Figure 3) in some ways appears to exclude functions necessary for cell vitality for these enzymes. Since they appear exclusively at a specific site it seems logical that their functions somehow are interconnected.

Lysosomal enzymes, including AcidPase, are believed to control the release of cell adherence (9,33,37-39). The ability to release cells from surrounding structures without cell destruction and to increase cell mobility would seem to be advantageous in a stretched tissue such as junction mucosa of the oral sphincter and may involve not only AcidPase but also AMPase and AlkPase.

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CHARACTERIZATION OF ALKALINE PHOSPHATASE FROM DEVELOPING
RAT BUCCAL MUCOSA

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ABSTRACT

Alkaline phosphatase (AlkPase) is a plasma membrane integrated protein of unknown function and found in parenchymatous and mineralized tissues. It is normally not present in squamous epithelium. Nevertheless it has been demonstrated in developing rat oral epithelium.

In this study the enzyme was solubilized from rat buccal mucosa and partially purified by gel chromatography followed by ion exchange chromatography. Three isozymes with different characteristics and sensitivities to heat inactivation were obtained. All three had a pH optimum of 10.2 and a Michaelis-Menten constant of 0.3 mM. Combined histochemical analysis of electrophoretic AlkPase zymograms and freeze-dried tissue sections indicate that the two most heat resistant AlkPase isozymes were of epithelial origin and that the third originated from the endothelium.

INTRODUCTION

Non-specific alkaline phosphatase (AlkPase) is an enzyme or group of enzymes generally found in many tissues of the body such as parenchymatous organs like the liver and kidney, in mineralized tissues, in the intestines, in the placenta, in the secretory epithelia and in the endothelium of the vascular tree (1,8,14,17). It is normally not found in squamous epithelium. In new born rats however, the enzyme is unexpectedly found in the squamous epithelium of the whole oral cavity and also in the anal and vaginal opening epithelium. With increasing age the enzyme activity of the oral cavity diminishes and is confined only to a junction type of epithelium which is found in regions where different type of mucosa merges. Such a region, which in the 10-day-old rat is particularly rich in its content of AlkPase activity, is the foldlike structure of buccal mucosa, which extends from the incisors of the upper jaw via the commissural area to the lower incisors (32,33).

Generally AlkPase has been shown to be a plasma membrane integrated protein and suggested to maintain functions in relation with transport of larger molecules through membranes (10,12,16,17), a function possible also for the enzyme in rat oral epithelium. However, recent studies of AlkPase and other enzymes such as acid phosphatase (AcidPase) and 5'nucleotidase (adenosine monophosphatase AMPase) of developing rat oral mucosa (32,33) indicate that other functions for mucosal AlkPase should also be considered and that the functions of the other mucosal phosphatases may be related with that of AlkPase. In order to clarify their interrelations it would be necessary to know the characteristics of the AlkPase for comparisons with other phosphatases.

Biochemical studies have shown that tissue bound AlkPase can be extracted and separated by gel filtration and ionexchange chromatography (13,17,37) and by poly-

acrylamide gel electrophoresis (15,17). The method of adding solvents active against phospholipids of plasma membranes, e.g. butanol (28,29) was introduced in order to increase the solubility of membrane bound AlkPase. Since then more efficient non ionic detergents like Triton X-100 have been introduced with the extraction medium and with gels in the electrophoretic separation techniques to improve the solubility of membrane bound proteins (3,9,15,20,21,22,35).

The purpose of the present study was to solubilize AlkPase from mucosal extract and to purify the enzyme by gel filtration and ion exchange chromatography. Purified fractions with AlkPase activity were analyzed for pH dependence, optimal substrate concentration and for sensitivity to heat inactivation. For corroborative purposes AlkPase of mucosal extract was also analyzed with electrophoresis followed by heat treatment. Also freeze-dried tissue sections of rat buccal mucosa were exposed to heat and the results from the three methods compared. For reference purposes in the electrophoretic analysis extracts from bone was also included.

MATERIAL AND METHODS

Ten-day-old Sprague-Dawley rats were killed and their buccal mucosa and bones carefully dissected out. The tissues were homogenized 3 times for 15 seconds in 5 volumes of 0.1 M Tris-HCl, pH 7.4, containing 1 % Triton X-100 and left overnight at +4°C. Extraction without Triton X-100 gave a low yield of AlkPase hence Triton was used in the extraction medium throughout. The tissue suspensions were ultracentrifuged at 105 000 g for 60 minutes at +4°C and samples of the supernatants were collected for the electrophoretic procedures described below. The remainder of the supernatant was dialyzed overnight against "S-200 buffer" (0.1 M Tris-HCl pH 7.4 containing 0.5 M NaCl, 1% Triton X-100, 1 mM MgCl₂, 1 mM CaCl₂ and 0.2 mM ZnCl₂). The dialyzed enzyme

solution was subjected to gel chromatography on a Sephacryl S-200 Superfine^R column as described in the legend to Fig. 1. The AlkPase containing samples obtained with S-200 Sephacryl were pooled and dialyzed overnight against 0.1 M Tris-HCl, pH 9.2 and subjected to chromatography on DEAE⁺-Sephacryl^R as described in the legend to Fig. 2. The three different peaks of AlkPase obtained with DEAE⁺-Sephacryl were pooled separately.

Enzyme characterization

Samples of the homogenate, the supernatant and the fractions from the columns were assayed for AlkPase activity with 125 μ l enzyme solution in 875 μ l 0.05 M carbonate-bicarbonate buffer, pH 10.0, containing 0.75 mM p-nitrophenyl-phosphate (p-NPP) and 10 mM MgCl₂ at 37°C in a waterbath. Hydrolysis of p-nitrophenol was determined from the optical density at 410 nm. The assays for determining the Michaelis-Menten constant of purified AlkPase were conducted with substrate (p-NPP) concentrations varying from 0.1 mM to 5 mM at +37°C in 0.05 M carbonate-bicarbonate buffer, pH 10.0 containing 10 mM MgCl₂. The assay was stopped and the colour developed by addition of 1 ml 1 M NaOH. Determination of pH optimum was done with 0.3 mM p-NPP and 10 mM MgCl₂ dissolved in 0.05 M carbonate-bicarbonate buffers of varying pH ranging from 9.0 to 10.7. Protein was determined according to Lowry et al. (25).

Electrophoresis procedures

Polyacrylamide gel rods for the electrophoresis were cast in glass tubes of 5 mm inner diameter and of 65 mm length and placed vertically in a Pharmacia electrophoresis system. Succrose, to a final concentration of 15% was added to the samples. Twenty μ l of each sample was carefully layered on top of the rods and under the buffer solution. The polyacrylamide gel system was a 7.5% acrylamide, pH 8.3 of Davis (11) modified by Maurer (26) and

by Fishman (15) who suggested addition of Triton X-100 (0.5%) to the gel. The Triton X-100 concentration in this study was lowered to 0.25% due to difficulties in casting the gels. The upper electrode reservoir contained with 0.1 M Tris-glycine buffer, pH 8.3. Bromphenol blue was added to the upper reservoir in order to stain the proteins of the migration front. The electrophoresis was run for the first five minutes with 1mA/gel and after that with 2mA/gel at a constant voltage. The electrophoresis was stopped after 5 hours or when the bromphenol blue stained migration front had reached the end of the gel rods. The gels were removed from the glass tubes, briefly rinsed in distilled water and incubated for AlkPase.

Histochemical procedures for sections and gels

One ten-day-old rat from the same litter as used for the enzyme characterization was anaesthetized with ether and decapitated. The head was mounted on a microtome stage and embedded in carboxy methyl cellulose dispersed with water and frozen to a block in hexane cooled with dry ice to -75°C . Horizontal sections were cut from the block at 20 μm interval, freeze-dried and transported to room temperature in an airtight box in order to avoid condensation artefacts (38). Freeze dried sections and gels from the electrophoretic procedures were left in $+56^{\circ}\text{C}$ distilled water for 10, 20 and 30 minutes and some sections also for 60 minutes. Untreated and heat inactivated sections and gels were then incubated for AlkPase. The sections were incubated, according to a method described by Burstone (7) with modifications, in 0.3 mM Naphtol-As-Mx-phosphahte as substrate and Fast Blue RR as coupling salt dissolved in 0.2 M Tris HCl, pH 8.3. The gels were incubated in 1 mM -naphtyl acid phosphate, 1.5 mM 4-amino-diphenylamide and 2.5 mM $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ dissolved in 0.25 M Tris maleate buffer, pH 9.2 (4,36). The gels and sections were incubated for 15-30 minutes at

room temperature. The incubation was stopped by rinsing the sections and gels in distilled water at +4°C. The sections were mounted in glycerine jelly. The gels and the sections were analyzed immediately and then photographed.

Materials All chemicals were of analytical grade and purchased from Sigma Chemical Co., USA, except Sepharcryl S-200, DEAE-Sepharose and the chromatographic and electrophoretic equipment were all obtained from Pharmacia Fine Chemicals, Sweden.

RESULTS

Enzyme characterization

The crude mucosal extract contained 9.9 mg protein/ml with a specific AlkPase activity of 0.8 $\mu\text{mol pNPP/h/mg}$ protein. The supernatant after ultracentrifugation contained 70% of the original AlkPase activity. Gel chromatography on Sephacryl S-200 gave a single AlkPase peak eluting after the void volume (Figure 1). The Sephacryl S-200 pool was separated into three fractions with DEAE⁺-sepharose ion-exchange chromatography (Figure 2). All three pooled fractions contained approximately 0.04 mg protein/ml. They had specific AlkPase activity of 2.6, 6.8 and 0.6 $\mu\text{mol pNPP/h/mg}$ protein for peak one, two and three, respectively (Table I). Heat inactivation (+56°C and 35 minutes) resulted in 30%, 65% and 85% inactivation of samples of peaks one, two and three respectively (Figure 2). All three peaks showed the same Michaelis-Menten constant (0.3 mM p-NPP at pH 10.0) and the same pH optimum (pH 10.2).

Electrophoresis

Bone extract AlkPase zymogram consisted of one distinct and intensely stained slow migrating band and

another diffuse fast migrating band which stained weaker and was inactivated by heat treatment. Oral mucosa AlkPase zymogram consisted of three bands. Of the two slow migrating distinct bands the faster stained intensely and the slower stained moderately. The third was a diffuse fast moving and weakly stained band which was sensitive to heat (Figure 4).

Histochemistry

Intense AlkPase staining was seen in the epithelium of the buccal fold and the lateral parts of the tongue. The staining was concentrated to the spinosum and granulosum layers. No staining was seen in the basal layer. In the border regions between AlkPase active and inactive cells of the epithelium the staining appeared randomly among the cells. In the connective tissue only the fine capillaries of the mucosa were stained for AlkPase and to a moderate extent. Tooth forming and bone forming cells showed intense staining of AlkPase while nerves and salivary glands were moderately active.

Heat inactivation of tissue sections showed that capillaries of the oral mucosa was completely inhibited after 30 minutes in $+56^{\circ}\text{C}$, but slight inhibition was observed already after 10 minutes (Figure 5b). After 1 hour treatment some AlkPase activity was still seen in the buccal fold epithelium and in the reduced enamel epithelium of the tooth forming tissues. All other tissues of the head were inactive for AlkPase.

Control incubations without the substrate were always negative, both in gels and in tissue sections.

DISCUSSION

The proteins with AlkPase activity that eluted from the Sephacryl S-200 in one peak were separated from plasma membrane residues and larger protein complexes

which left the column with the void volume. This and other findings such as the ultrastructural finding of plasma membrane localization of mucosal AlkPase (Sjögren and Larsson unpublished results) and the need for a detergent to solubilize the enzyme shows that mucosal AlkPase in these respects seems no different from AlkPases of other tissues of the body (8,12,13,14,16,17).

Since the exclusion limit for Sephacryl S-200 is approximately 250 000 D for globular proteins (Pharmacia specifications), the AlkPase activity seems to have been associated with one or several proteins with a total molecular weight of less than 250 000 D, a finding which is in accordance with previous reports on the molecular weight of AlkPase from different tissues and species and the dimere of which range from 130 000 - 200 000 D (5,17,18). However, the extraction method used in the present study may have induced some systematic errors. The mucosal extract was left overnight with the detergent present in the extraction medium in order to solubilize the enzyme from the plasma membranes and solubilized proteases and lysosomal enzymes may have acted on the AlkPase molecules. In such a fluid an equilibrium between monomeric and polymeric proteins of the same family may occur. Since AlkPase is known to exist in multiple forms, where the dimere is believed to be the native form of the enzyme *in vivo* (17,34) one has to consider the possibility that the three fractions later obtained represented different polymers of AlkPase rather than different AlkPase isozymes. But then they would have left the S-200 column in separate fractions and not in a single peak as was the case.

All three fractions obtained with DEAE-sepharose chromatography had characteristics that compare well with previous results obtained for AlkPase of different tissues and species in general (8,14,17,18) and their different resistance with regard to heat inactivation indicate that they represent true AlkPase isozymes (30).

The mucosal homogenate contained material from both epithelial and connective tissue cells and the results seemed to corroborate the findings of the chromatographic procedures with three different AlkPase isozymes in rat oral mucosa. The heat inactivation study of the ion-exchange chromatographic and electrophoretic preparations and of the tissue sections showed that one of the AlkPases was more sensitive to heat inactivation than the other two and probably of endothelial origin.

The bone homogenate contained two AlkPase isozymes with different sensitivity to heat inactivation. This finding is different from some early studies (19,30), but in accordance with more recent done on as well bone (1,6,15,31) as of other mineralized tissues like cartilage (2) and pulp (20). In the present study the fast moving band of bone AlkPase had migrated the same distance as the endothelial AlkPase of mucosa and also showed the same diffuse staining and sensitivity to heat inactivation. The bone preparation contained marrow tissue including endothelial cells. It is thus possible that the heat labile fast moving band of bone was of endothelial origin.

The function of the two AlkPases of the oral epithelium is unknown. However they appeared in a region of the oral mucosa where other phosphatases like AMPases, and AcidPases have been found (32,33). Counting the present study it seems as if the squamous epithelium of developing rat buccal fold contains at least two AlkPases, two AMPases and three AcidPases (32,33). The biological significance of the complex gathering of phosphatase activities may be interpreted in several ways. The phosphatases may be connected with the keratinization process of oral squamous epithelium, but since the entire mucosa is keratinized while the enzymes appear only at selected regions, such an explanation is incomplete. A second possibility is based on the findings of Jarrett and associates (23) who identified a variety of

phosphatases with an alkaline pH in squamous epithelium of rat skin exposed to retinyl acetate. Jarrett (24) suggested that the phosphatases were connected with regulatory mechanisms of cellular growth, functions which coincides well with that of cellular differentiation suggested for intestinal AlkPase by Moog et al. (27). Such functions seems feasible also for phosphatases of developing rat oral epithelium. But, since the phosphatase activities were found only at specific regions where the mucosa has to meet increased demands from stretching forces as compared with neighbouring areas, then other functions directly connected with the stretching phenomenon, such as maintenance of epithelial cell attachment or cell membrane renewal, may also be considered.

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Figure 1. Chromatography of alkaline phosphatase from rat buccal mucosa.

Two ml of supernatant from approximately 1g of mucosa (see material and methods) was chromatographed at +4°C on a column (83.3 x 1.6 cm) of Sephacryl S-200 in "S-200 buffer". The flow rate was 78 ml/hour and 1 ml fractions were collected. The fractions were assayed for AlkPase activity (see material and methods). The void volume of the column (V_0) was determined with Dextran blue to be 65 ml and the total column volume (V_T) was 167 ml.

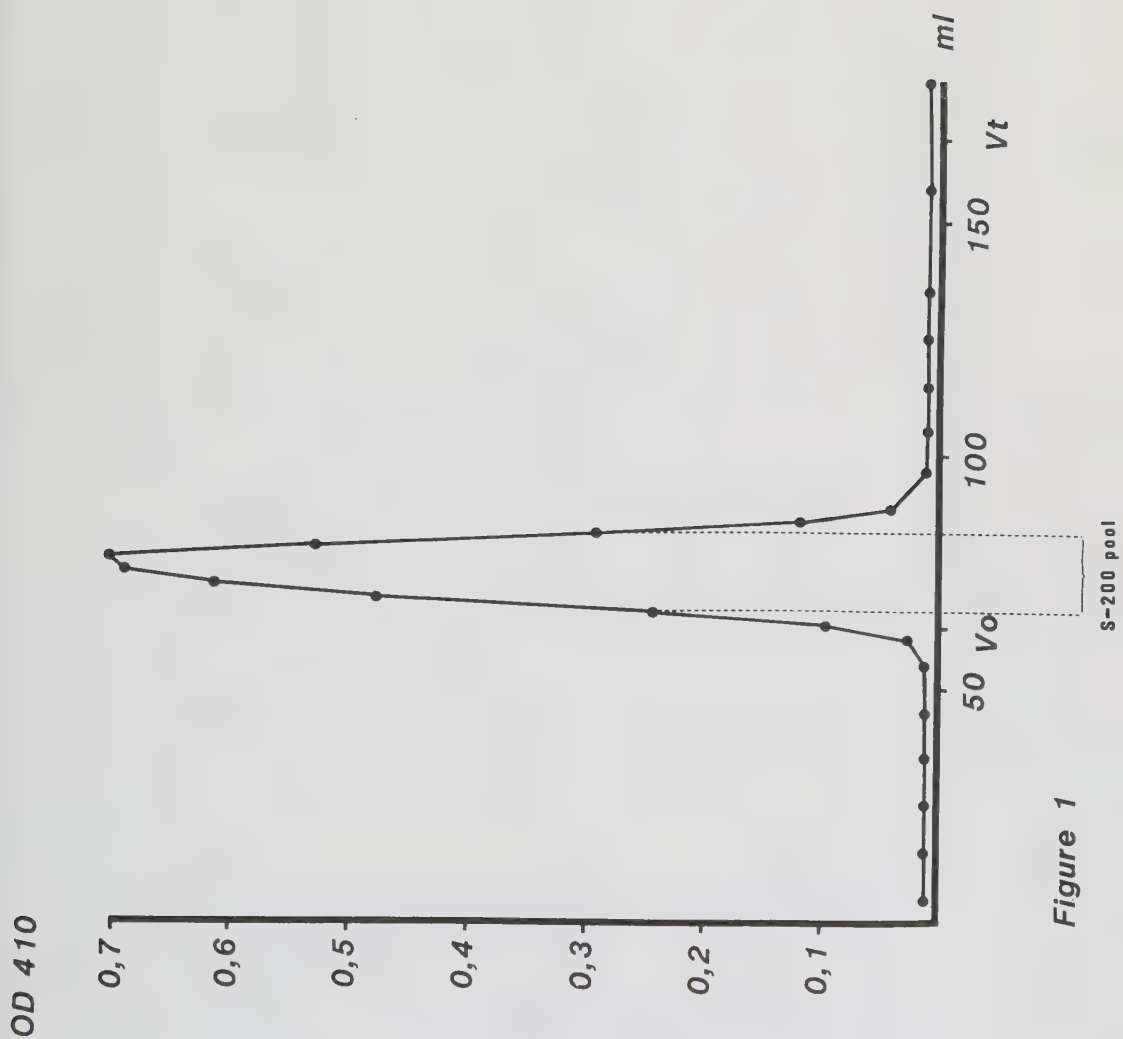


Figure 1

Figure 2. DEAE⁺-Sephadex column chromatography of 10-day-old rat mucosa alkaline phosphatase S-200-pool. The S 200-pool (10 ml) was loaded on a DEAE-Sephadex column (26x0.9 cm) after dialyzing over night against 0.1 M tris-HCl, pH 9.2. The same buffer was used to equilibrate the column with. The column was eluted with 100 ml of 0.1 M tris-HCl buffer, pH 9.2" followed by a linear gradient of NaCl from 0.0 to 1.0 M in 400 ml of the same buffer.

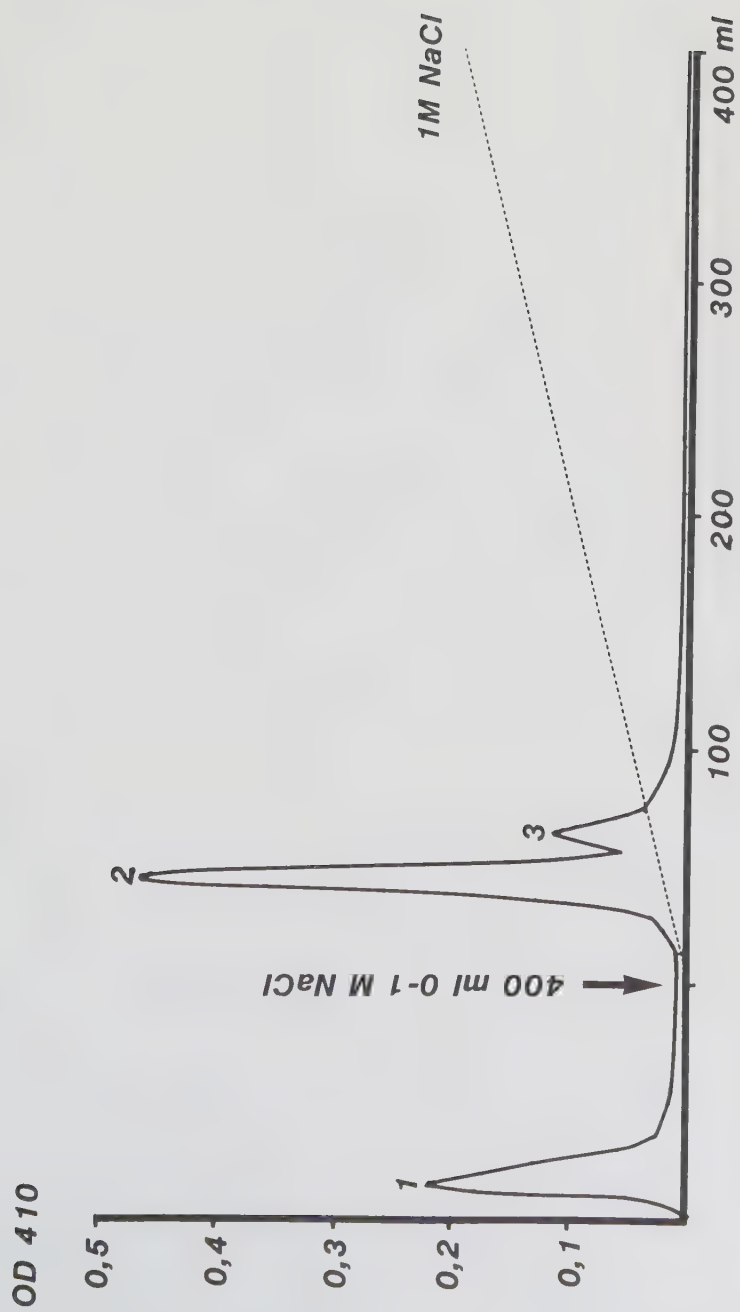


Figure 2

Figure 3. Heat inactivation of AlkPase fractions obtained after the ion exchange chromatography. ○—○ = AlkPase from peak 1, ●—● = AlkPase from peak 2 and ■—■ = AlkPase from peak 3.

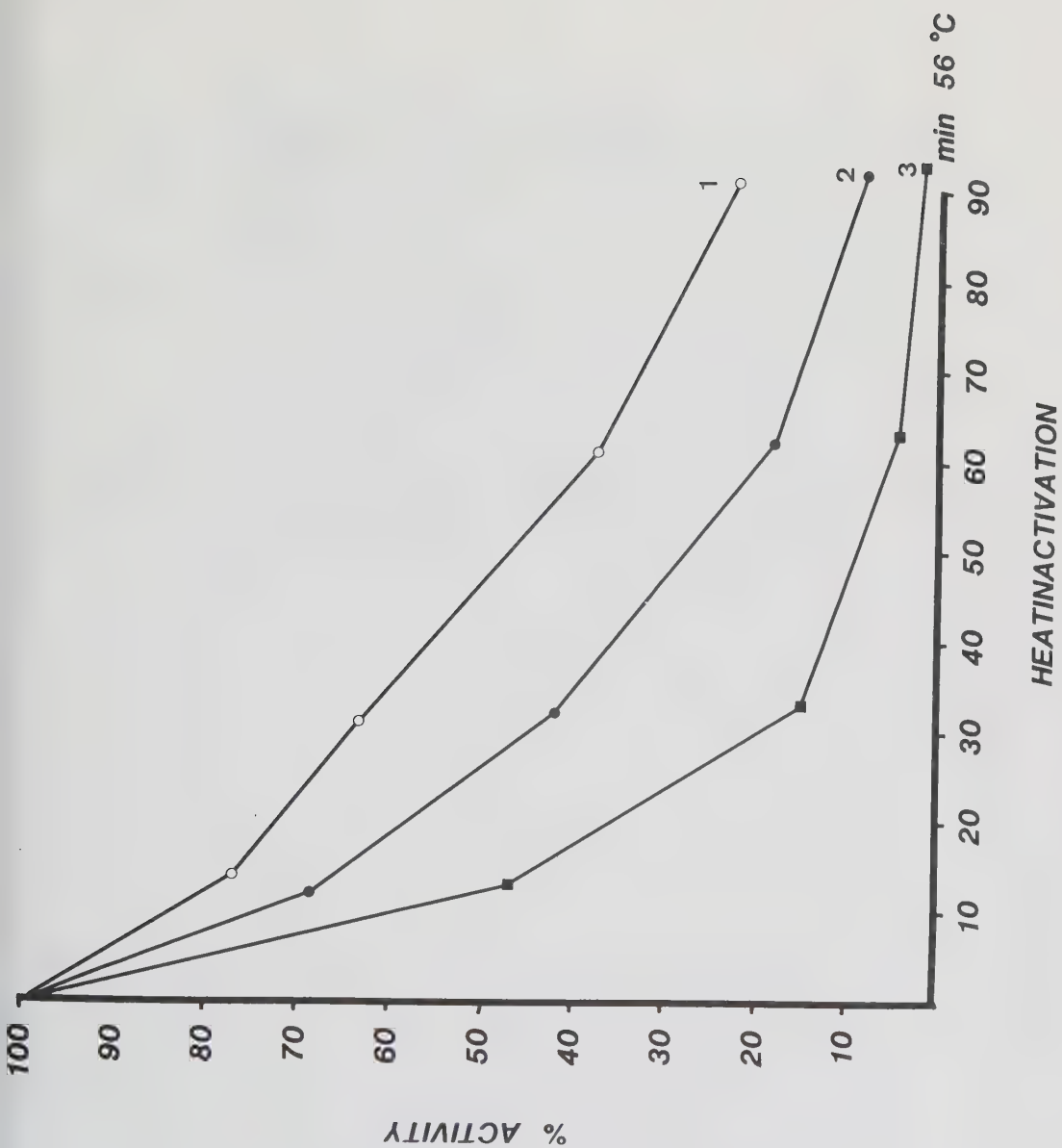
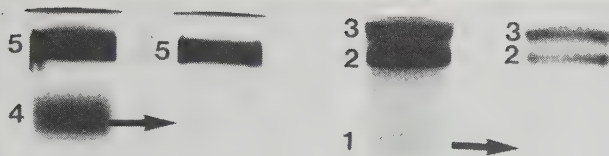


Figure 4 shows the effect of heat inactivation on AlkPase isozymes from bone (A,B) and oral mucosa (C,D) obtained with polyacrylamide gel electrophoresis. (The gels were incubated for AlkPase according to the method of Taswell and Jeffers (1963) modified by Beck et al.(1975)) The gels A and C were kept at room temperature while gels B and D were kept at +56⁰C for 20 minutes.



BONE

ORAL
MUCOSA

—

56°C

—

56°C

A

B

C

D

Figure 5a-c. Freeze-dried serial sections from the buccal fold region of a 10-day-old rat. The sections were incubated for AlkPase at pH 8.3 according to the method of Burstone (1958) and show the effect on AlkPase activity of heat inactivation in +56°C for 0-20 minutes.

5a. No heat inactivation done on this section. Note intense AlkPase staining in all layers of the epithelium except the basal layer (arrowheads). Moderate staining is also seen in the capillaries of the connective tissue (arrows). No staining is seen in the connective tissue (CT) or the muscular tissue (M).

5b. Section heat inactivated for 10 minutes. Moderate AlkPase staining remains in the epithelium and weak staining can still be seen in the capillaries (arrows).

5c. Section heat inactivated for 20 minutes. Weak to moderate AlkPase staining is seen in the epithelium while all endothelial AlkPase activity in the capillaries is inactivated (arrows).

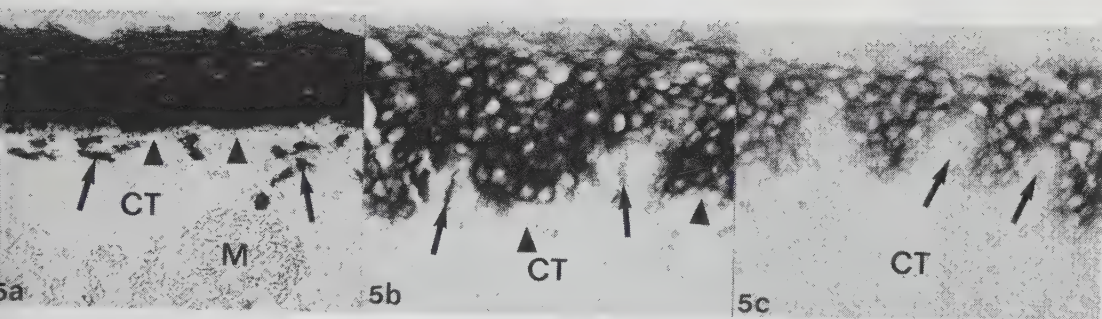


TABLE 1. Partial purification of alkaline phosphatase from rat buccal mucosa.

Fraction		Protein (mg/ml)	Specific activity ($\mu\text{mol pNP/h/mg protein}$)
Crude-extract		9.9	0.8
S-200-pool (=DEAE-load)		0.6	0.5
DEAE ⁺ - pool	1.	0.037	2.6
	2.	0.043	6.8
	3.	0.045	0.6

^{65}Zn UPTAKE AND ALKALINE PHOSPHATASE IN DEVELOPING RAT
ORAL MUCOSA.

By

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Running title: ^{65}Zn and alkaline phosphatases in rat
mucosa.

Key words: Alkaline phosphatase, autoradiography, epi-
thelium, histochemistry, rat, $^{65}\text{Zinc}$.

ABSTRACT

Alkaline phosphatases (AlkPase) of many different tissues and species have been shown to be zinc metallo enzymes. Specific regions of rat oral mucosa contains AlkPases and they also retain injected radioactive zinc (^{65}Zn).

The relationship between mucosal AlkPases and retained ^{65}Zn was analyzed by comparing the uptake of ^{65}Zn in autoradiographic plates of rat mucosa with AlkPase staining of the corresponding areas in freeze-dried tissue sections. EDTA mediated inactivation of AlkPase and reactivation with zinc and magnesium was also investigated. Mucosal extract containing ^{65}Zn and AlkPase activity was separated on polyacrylamide gel electrophoresis and the gels analyzed for radioactivity and for AlkPase activity.

It was found that mucosal AlkPase is zinc dependent and possibly a zinc metallo enzyme. It could not be verified with electrophoresis and combined radioactivity measurements that the ^{65}Zn uptake of oral mucosa was incorporated with the AlkPase molecule.

INTRODUCTION

Alkaline phosphatases (AlkPase E.C.3.1.3.1.) are a group of enzymes of unknown function found in many different type of tissues. They are normally not found in squamous epithelium although they are richly present in resorbing or secretory epithelia. In young rats, however, AlkPase is found in the oral squamous epithelium, particularly in a fold like structure where different types of mucosa merges. This fold is situated in the anterior part of the buccal mucosa and extends from the incisor region of the upper jaw to the incisor region of the lower via the commisural region. This mucosa differs from other parts of the oral mucosa by its content of AlkPase and also of 5'nucleotidase (adenosine monophosphatase, AMPase E.C. 3.1.3.5.) (30, 31,32).

AlkPase isolated from several other tissues also of different species has been shown to be zinc metallo enzymes (20,26,28,34). The molecule contains four zinc atoms two of which are situated in or very near the catalytic site of the enzyme. The other two are structural parts of the molecule and without significant effect on the catalytic activity (7,26). In an autoradiographic study of the uptake and distribution of radioactive zinc (^{65}Zn) in young rats a distinct uptake in the anterior parts of the oral mucosa was described by Bawden and Hammarström (4).

In biological systems zinc is an essential trace element. In mammals it is thought to be absorbed in the intestinal mucosa, bound to a protein and distributed to various target tissues, where it is believed later to appear in e.g. enzyme systems (for review see 7). Zinc deficiency is known to cause pathological changes in various tissues (9). Deficient animals develop, among other defects, parakeratotic lesions in the oral mucosa and the skin with thickening of epidermis and intra and intercellular oedema and increased mitotic and metabolic

activity (1,2,3,14,15,23,25,29). This points to a zinc dependance of keratinizing epithelial cells (37) but the function of zinc in epithelium is unknown.

The purpose of the present paper was to study the relation between zinc and AlkPase acitivity in the oral mucosa of developing rats. This was done by; 1. comparing the localization of injected ^{65}Zn with that of AlkPase in the mucosa; 2. analyzing the presence of injected ^{65}Zn in AlkPase molecules; 3. analyzing the effect of EDTA treatment and reactivation with zinc on AlkPase activity in tissue sections from buccal mucosa of young rats. For comparisons AMPase and magnesium analysis were included in this part of the study.

MATERIAL AND METHODS

Autoradiography

Eight 4-day-old Sprague Dawley rats were each given intraperitoneal injections of 10 μCi ^{65}Zn every day, four consecutive days, and then left for an additional two days. Three of the 10-day-old rats were then anaesthetized with ether and embedded on a microtome stage in a mixture of carboxymethyl cellulose and rapidly frozen (-75°C). Whole body horizontal, sagittal and frontal sections (20 μm) were cut and freeze dried. The sections were pressed against Structurix X-ray film (AgfaGevaert) in a dark room and left for exposure (35,36). After one week the filmplates were separated from the sections, developed and analyzed.

Enzyme histochemistry

The autoradiographed sections were incubated for AlkPase in 0.09 mM Naphtol-As-Mx phosphate as substrate and Fast Blue RR as coupling salt dissolved in 0.2 M Tris-HCl, pH 8.3 (8). The sections were incubated for 30 minutes at $+37^{\circ}\text{C}$ and the reaction stopped by washing in distilled water. Other fresh cut freeze-dried sections were incubated for AlkPase activity in 10 mM B-glycero-phosphate as substrate in 0.08 M Tris-HCl, pH 10.0, with 3.9 mM MgCl_2 and 2 mM lead citrate (22). These sections were incubated at $+37^{\circ}\text{C}$ for 15 minutes and the reaction stopped by washing in 0.9% NaCl. The staining was then developed in 1% ammonium sulphide.

Fresh cut freeze-dried sections were treated in 30 mM EDTA for 48 hours at $+4^{\circ}\text{C}$ (12,18,24) some of which were reactivated either with 0.5 mM ZnCl_2 or 1 mM MgCl_2 before incubation for AlkPase. In addition EDTA treated sections with and without zinc or magnesium reactivation and untreated sections were also incubated for AMPase activity in 3.6 mM adenosine monophosphate, 3.4 mM lead nitrate and 10 mM MgCl_2 dissolved in 0.08 M Tris-ma-

leate, pH 7.2 (19,39). In order to inhibit nonspecific AlkPase activity 0.1 mM L-p-bromotetramisole was added to the incubation medium as recommended by Borger and Thoné (6). The sections were incubated for 15 minutes at +37°C, washed in 0.9% NaCl and the staining developed in 1% ammonium sulphide. All sections from AlkPase and AMPase histochemistry were mounted in glycerine jelly, analyzed and photographed.

Electrophoresis

The remaining five rats were decapitated and the buccal folds of the oral mucosa were rapidly dissected free and homogenized in a glass homogenizer with 5 volumes of 0.1 M Tris-HCl buffer, pH 7.4, with addition of 1% Triton X-100. The crude extract was centrifuged (40 000g x 30 minutes) to free the protein solution of whole cells and cell fragments. The supernatant was carefully pipetted off and mixed with an equal volume of 40% sucrose. Thirty μ l of the extract-sucrose mixture was carefully layered on top of a separation gel in a 7.5% polyacrylamide disc. electrophoretic system, pH 8.3, of Davies (10) with modifications (11,21,32). The electrode reservoirs were filled with 0.1 M Tris-glycine, pH 8.3, with bromphenol blue added to the upper chamber to stain the migration front. The electrophoresis was carried out at a constant voltage with 2.0 mA/gel and ran for five hours.

Radioactivity measurements

The distribution of ^{65}Zn in the gel rods was studied by dividing the gel into 2 mm thick slices. Each slice was placed in a special plastic tube for radioactivity measurements with a NaI(Tl) well crystal in a Selectronic sample changer. ^{65}Zn decay through electron capture (98.3%) and positron emission (1.7%) with a physical halflife of 245 days. The photon radiation emitted is Cu-X-rays, annihilation photons 0.511 MeV

(3.4%) and gamma radiation 1.115 MeV (49%) and measured in the high energy channel. A background activity of 0.17 counts/second was recorded throughout the gels with a coefficient of variation in the net counts of 15% and a total of 3.3 counts/second for the whole gel.

Zymogram staining

The AlkPase distribution in the gels was studied by incubating the gels in 1 mM -naphthyl acid phosphatase, 1.5 mM 4-aminodiphenylamide and 25 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 0.25 M Tris-maleate buffer, pH 9.2 (5,33). One gel was used as control and was incubated without the substrate.

Controls

Control incubations of sections were included regularly where the substrate or the capturing ion was substituted for distilled water.

Materials

Zinc chloride with an activity concentration of 185 MBq/ml (5 mCi/ml) was purchased from New England Nuclear, Boston, USA. All other chemicals were of analytical grade and obtained from Sigma Chemical Co., Missouri, USA.

RESULTS

Autoradiography and histochemistry

Two days after the last of four consecutive daily injections of ^{65}Zn , the blood level uptake, as seen in the heart lumen, was very low, while a high uptake of ^{65}Zn was seen in the teeth and bones. Uptake nearly as high as in mineralized tissues was also found in the liver and intestines while a low uptake was seen in the kidney cortex (Figure 1a). A moderate uptake was present in epithelial cells of the oral mucosa. The ^{65}Zn uptake was localized at the anterior parts of the oral cavity mucosa and it was associated with the foldlike structure of the buccal mucosa (Figures 1c and 2,d,e,f). Sections first autoradiographed and then incubated for AlkPase showed a distinct staining pattern of the epithelium in junction mucosa, which corresponded well with the uptake patterns of ^{65}Zn . The staining was distributed among the cells in the spinosum and granulosum layers. In the area bordering to unstained parts of the mucosa, the staining of the cells became gradually randomized and mixed with non-stained cells. No staining was seen in basal cells. In the connective tissue cells, subjacent to the epithelium, staining of capillaries could also be seen while the connective tissue fibres and cells was more or less unstained (Figures 1 b and c and 2a,b and c). However, some areas of the mucosa without any visible sign of AlkPase activity, showed uptake of ^{65}Zn and areas with intense AlkPase activity lacked uptake of ^{65}Zn (Figures 2b,c and 2e,f). Also in teeth ^{65}Zn was accumulated at the mineralized parts of the dentin/enamel tissue but little or none was seen in the pulp and enamel organ, where intense activity of AlkPase was found (Figures 1b,c). The parotid gland also contained AlkPase activity without any sign of zinc accumulation (Figures 2b and e).

Sections stained for AMPase showed activity in the same regions which contained AlkPase activity. In addi-

tion the basal layer of large areas of the oral cavity showed AMPase staining. Capillaries of the subepithelial connective tissue stained slightly for AMPase activity. Treatment in 30 mM EDTA for 48 hours extinguished all AlkPase and AMPase activity in the epithelium. Reactivation of EDTA treated sections with zinc restored the activity of AlkPase and AMPase, while addition of magnesium restored only a minimum of the activity (Table I).

Electrophoresis

Extracts of oral epithelium from ^{65}Zn injected rats subjected to disc. electrophoretic separation on acrylamide gels contained three fractions which stained for AlkPase. The proteins that were AlkPase active had migrated into the gel and formed three bands of which the one nearest the anode stained moderately and the next intensely. Both these were narrow and distinct and had migrated only a short distance into the gel. A diffuse, third band which stained weakly had moved a considerably longer distance into the gel.

The ^{65}Zn assay showed two significant peaks of different magnitude, with 0.8 counts/second in the larger and 0.3 in the smaller (Figure 3). The coefficient of variation in the net counts for the two peaks was only 2%. Bromphenol blue staining of the migration front proteins showed that the smaller ^{65}Zn peak corresponded with what was estimated to be the albumin/prealbumin region and the larger ^{65}Zn peak with the electrophoretic migration front.

Control incubations without the substrate present were done regularly and were negative.

DISCUSSION

The autoradiographic findings in this study with uptake of ^{65}Zn in mineralized tissues, liver, intestines, kidney and oral mucosa and a very low blood level are in

good agreement with previous results obtained also on the developing rat (4). Also the AlkPase activity pattern of the oral mucosa found in the histochemical and electrophoretical analysis corresponds with previous reports (30,31,32). Furthermore, the blood level of ^{65}Zn , as indicated by the uptake seen in the heart lumen, was very low and hence interference from the blood tissues seems to have been negligible. It is thus probable that there was a genuine retention of ^{65}Zn in epithelial cells. The concentration of ^{65}Zn to the anterior parts of the oral mucosa corresponded suggestively to the AlkPase activity found in the same regions of the oral epithelium. However, this similarity in distribution was not complete in every region and it is therefore doubtful if the accumulated zinc really reflected zinc that had been incorporated with the AlkPase molecule. In the teeth and the parotid gland this lack of correspondence between AlkPase and ^{65}Zn accumulation was evident.

From the inactivation tests with EDTA it was found that AlkPase and AMPase were both sensitive to EDTA, which is a chelator and removes cations like calcium, magnesium, zinc etc. from different types of tissue. The inactivation of AlkPase and AMPase which followed the EDTA treatment is in accordance with previous results (12,16,27,31) and was shown to be overcome by addition of zinc but not by addition of magnesium. This may indicate that endogenous zinc was extracted from the enzymes and replaced by zinc from the medium, leading to restored activity. Alternatively another substance was removed from the enzyme and replaced with added zinc, which restored the activity. The fact that the catalytic activity could not be restored by magnesium addition seems to exclude the possibility that the reactivation of the enzyme was a non-specific cat ion effect but rather an effect of the zinc ion as described previously by Harkness (16) Yunis and Gentry (41) and PetitClerc et al. (27). It corroborates the supposition that oral mucosa

AlkPase is a zinc metallo enzyme like many AlkPases of other tissues (26). With AMPase the situation was the same.

Addition of L-p-bromotetramisole to AlkPase and AMPase incubation media showed that there are AlkPase and AMPase activities in oral epithelium which both are zinc dependant but reacts differently to L-p-bromotetramisole.

When analyzing the reaction between proteins and the retained ^{65}Zn in mucosal extract it was found that the majority of the retained ^{65}Zn had migrated in or very near the migration front. If ^{65}Zn had been incorporated with AlkPase in the epithelium it obviously had dissociated during the electrophoresis. The beginning and the end of the gel contained negligible amounts of ^{65}Zn indicating that no diffusion of ^{65}Zn from the peaks had taken place after the electrophoresis was finished. Thus the larger of the two ^{65}Zn containing peaks found in and near the migration front should represent dissociated and unbound ^{65}Zn or ^{65}Zn bound to proteins which migrated with the front. The smaller peak contained ^{65}Zn firmly bound to unknown proteins which migrated in or near the albumin region. The smaller peak was of such a low magnitude and scarcely above the background content that its existence must be questioned. Should it reflect a genuin zinc metallo-protein complex however it means that the binding energy between the zinc and the protein is very high and exceeds that of the electrophoretic forces applied. During normal purification procedures zinc metallo enzymes like AlkPase are not deprived of their zinc nucleus while metallo proteins where the metal atoms are more loosly bound, dissociates during electrophoresis (26,38).

In conclusion ^{65}Zn appeared only at specific regions of the oral mucosa. One may assume that the uptake was connected with some component(s) which also shows this regional distribution. The only substances with a similar regional distribution in the oral mucosa known to the

authors are AlkPase and AMPase. Both were shown in this study to be zinc dependant but they may not necessarily be zinc metallo enzymes. This is contrary to expectations (26). Further studies with alternative approaches or with gentler purification procedures than electrophoretic separation techniques are therefore needed to verify or to reject the assumption that AlkPase and AMPase of oral squamous epithelium are zinc metallo enzymes and to clarify the regional zinc retention of developing rat mucosal cells.

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Figure 1 a-c. Sagittal section of 10-day-old rat injected intraperitoneally with ^{65}Zn .

a. Autoradiogram of whole body section. Uptake is seen in mineralized tissues like bone (arrow-head) and teeth and also in liver (L), kidney (K) and intestines (I). Also note intense uptake in the anterior part of the oral cavity (arrow).

b. Detail from section used in the autoradiography in 1a, stained for AlkPase pH 8.3. Note intense staining of epithelium in the oral mucosa (arrow) which is part of the buccal fold. Also note staining of pulp (asterisc) and mineralized tissues (arrow head).

c. Detail of oral cavity from autoradiogram seen in 1a. Note intense uptake of ^{65}Zn in the anterior part of oral mucosa (arrow) and compare with the same region in 1b stained for AlkPase. Also note the lack of uptake of ^{65}Zn in pulpal cells of the molars (asterisc) and compare with intense staining for AlkPase in pulp tissue.

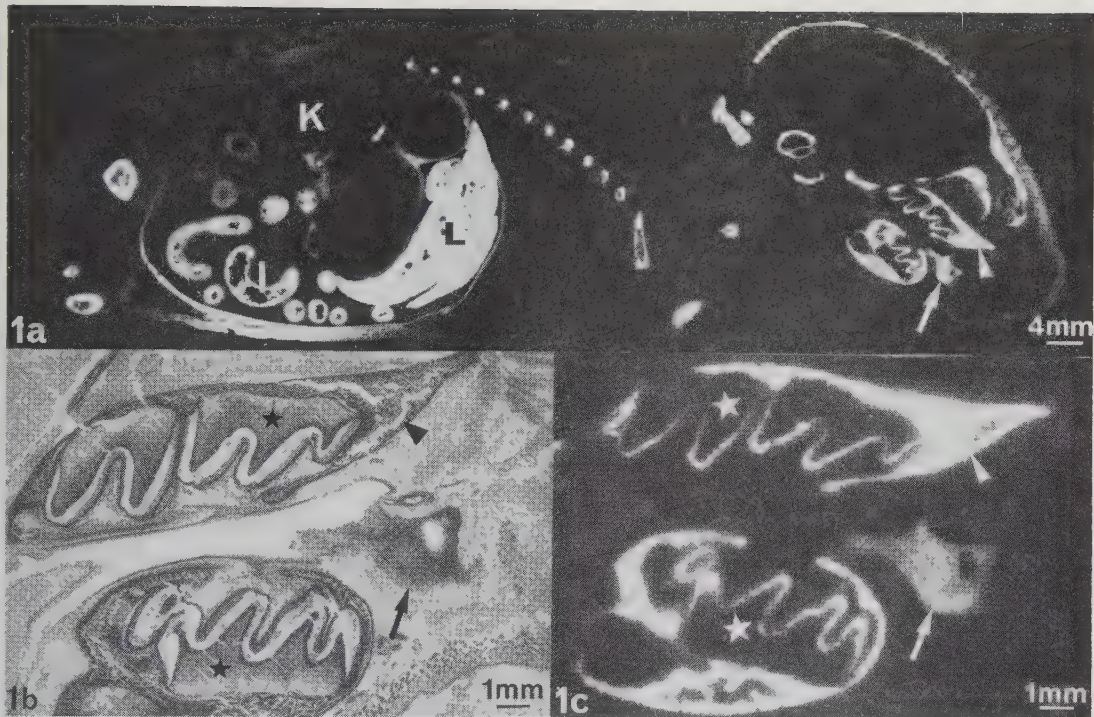
Figures 2a-f. Frontal, horizontal and sagittal views of 10-day-old rats. Neighbouring sections were used for AlkPase histochemistry and ^{65}Zn autoradiography.

a-c. Sections incubated for AlkPase, pH 8.3, with activity in anterior regions of oral epithelium (arrows and arrow head). Also note AlkPase activity in mineralized tissues (double arrows) and in the parotid gland (asterisc).

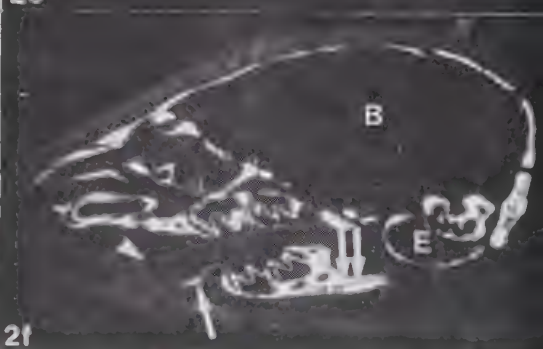
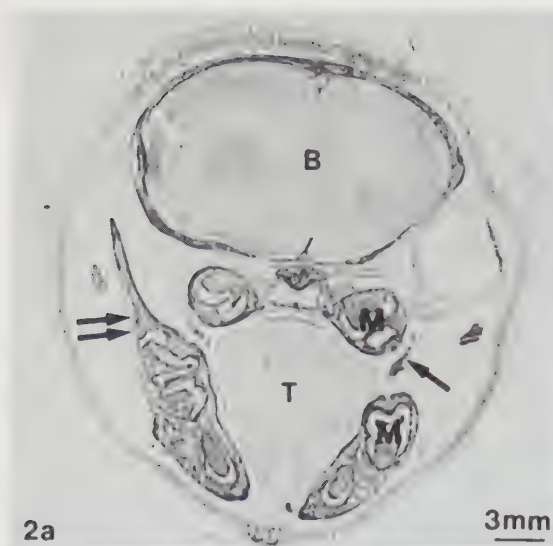
d-f. Autoradiograms of neighbouring sections to the one seen in a-c. The uptake of ^{65}Zn in the

oral mucosa corresponds well with the enzyme activity demonstrated in the oral epithelium in figures a-c (arrows). However in some areas of figure 2e (double arrow heads) ^{65}Zn uptake is evident but corresponding region in figure 2b shows no AlkPase activity (double arrow heads). Furthermore in other regions of figures 2e and 2f (arrow head) no uptake of ^{65}Zn is seen and yet the corresponding areas in the sections 2b and 2c show intense AlkPase activity (arrow head). The same finding is seen in the parotid gland with no uptake of ^{65}Zn (figure 2e asterisc) but intense staining of AlkPase (figure 2b asterisc). B=brain, E=inner ear, M=molars, T=tongue.

Figure 3. Graphic representation of AlkPase zymogram of buccal mucosa supernatant from 10-day-old rats which had been given repeated intraperitoneal injections of ^{65}Zn . The zymogram consisted of two distinctly and one diffusely stained AlkPase isozyme. The vertical arrow indicate the migration direction of the electrophoresis and the small arrow the migration front. Gels from the same electrophoretic run were sliced in two mm discs and assayed for ^{65}Zn radioactivity. The result is seen in the diagram to the right. The horizontal line indicate the radioactivity assayed in the individual discs. Two peaks of radioactivity were found (arrowheads).



1a-c



2a-f

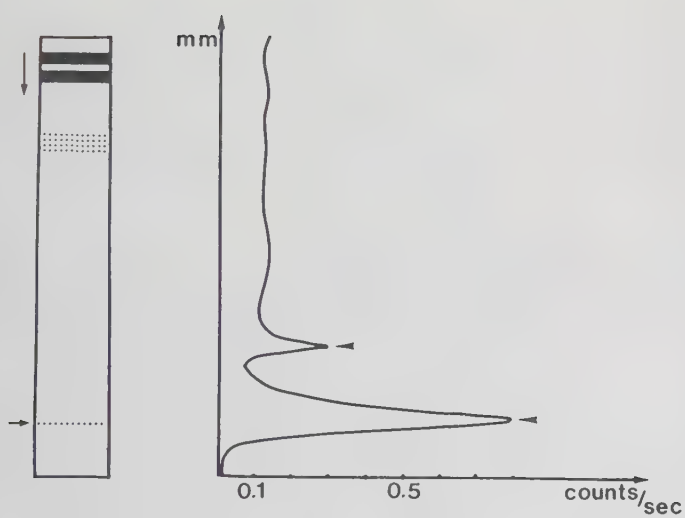


TABLE I

	EDTA treated		EDTA treated followed by Zn reactivation		EDTA treated followed by Mg ⁺⁺ reactivation	
	Basal cells	Spinosum granulosum	Basal cells	Spinosum/granulosum cells	Basal cells	Spinosum/granulosum cells
AlkPase 8.3	0	+++	0	+++	0	+++
AlkPase 10.0	0	+++	0	0	0	0
AMPase 7.2	++	+++	0	0	+	++

Table I. Semiquantitative evaluation of sections incubated for AlkPase, pH 8.3, (8), for AlkPase, pH 10.0, (22) and for AMPase, pH 7.2, (19,39) in order to show the effect of EDTA on AlkPase and AMPase activity and the restorative effect of zinc and magnesium on the enzyme activity. Note lack of response to EDTA in AlkPase activity at pH 8.3 and compare with AlkPase activity at pH 10.0. The coupling salt used for AlkPase activity detection at pH 8.3 contains zinc and reactivates the enzyme (17,31,40). +++= intens staining, ++= moderate staining, += low staining and 0= no staining detectable in sections.



Lactate Dehydrogenase in Developing Rat Oral Epithelium

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Freeze-dried sagittal, whole-body sections of 10-day-old rats were incubated for lactic dehydrogenase (LDH) using different media in the presence of the inhibitors urea and fluoropyruvate. Phenazine methosulfate (PMS) and menadione, which are regularly used in current histochemical media and are believed to promote the demonstration of LDH activity, were also added and shown to be insufficient for the demonstration of total LDH activity, and PMS even

seemed to have an inhibitory effect on LDH activity in oral epithelium. However, cumulated data from the different incubations show that the oral epithelium of developing rats may contain two different types of LDH, one in the basal cells with possibly aerobic characteristics, and another in the spinosum/granulosum cells with anaerobic characteristics.

KEY WORDS: Epithelium; LDH; Inhibitors; Isoenzymes; Rats.

Introduction

Lactate dehydrogenase (LDH) (E.C.1.1.1.27), a glycolytic oxido-reductase, catalyzes the interconversion of pyruvate and lactate in the presence of nicotinamide adenine dinucleotide (NAD), and is found in most tissues of many living organisms (for review see ref. 14). In the oral epithelium of humans (11, 13,18) and of rats (9,18), LDH has been demonstrated and found to be most active in the basal layer, with decreasing activity toward the surface. However, in a recent study of rat oral mucosa, a reversed LDH pattern was found (22) with a method described by Chayen et al. (4). Because there were major methodologic differences between this study (22) and the previous ones (9,11,13,18), it was suggested (22) that the seemingly divergent results in the different studies were caused by these differences. From the early era of LDH histochemistry, it has been recognized that there are technical difficulties to overcome in demonstrating LDH in tissue sections. One difficulty is the leakage of enzymes from tissue sections (6,19), which has been shown to lead to the false or imprecise localization of LDH. Corrections and refinements of the original histochemical procedures have therefore led to the introduction of tissue stabilizers such as agarose gel (21) and polyvinyl alcohol (PVA) (1), of semipermeable membranes (15), and of hydrogen acceptors such as phenazine methosulfate (PMS) (25) and menadione (16,17,23). Consequently, there are today several different histochemical methods available for LDH demonstration, each relying upon an identical basic histochemistry but employing tissue stabilizers and promoting the inhibiting substances of different composition (2,4,12,15,17,20). The purpose of the present study was to further analyze the

LDH activity of developing rat oral epithelium, and to study the effect of LDH-inhibiting and -promoting substances on this tissue. Whole-body sections were used to facilitate comparative studies between oral epithelium and other tissues.

Materials and Methods

Ten-day-old Sprague-Dawley rats were anaesthetized, frozen at -75°C , and sectioned with a cold microtome, as described by Ullberg (24). Serial sections $15\text{ }\mu\text{m}$ thick were taken at different planes and dried at -20°C . Prior to incubation, the sections were taken to room temperature in an air-tight box to avoid condensation artifacts.

Incubation Media. Medium 1. The basic medium for LDH was prepared according to Chayen et al. (4), and contained 22% PVA in 50 mM glycylglycine buffer (pH 8.0) to prevent the diffusion of reaction products and enzyme during incubation (1), and 50 mM D, L-lactate, 5 mM nitroblue tetrazolium (NBT), and 2.5 mM nicotinamide adenine dinucleotide (NAD), all added prior to degassing and N_2 -gas saturation. The following substances were also added, either alone or in various combinations (see below): phenazine methosulfate (PMS) at 0.2 mg/ml, to operate as a hydrogen acceptor and, mediator between reduced NAD and NBT, in order to counteract NADH-diaphorase interference (4,7,12,25); menadione (0.33 mg/ml), to obtain superior hydrogen mediation during aerobic glycolysis (16,17); and amobarbital (10 mM), to block interfering cytochrome *c*-oxidase (16); cyanide (1 mM), to inhibit the cytochrome oxidase system and to reverse an inhibitory effect of PMS (15) (see also Fig. 3). The resulting media had the following constituents:

- 1a. Basic medium + PMS + amobarbital
- 1b. Basic medium + menadione + amobarbital
- 1c. Basic medium + PMS + menadione + amobarbital

Table 1. Semiquantitative evaluation of LDH staining in basal cells of oral epithelium in sections incubated in media 1a–f, with and without inhibitors present in the medium^a

Medium	1a	1b	1c	1d	1e	1f
Inhibitor	PMS + A	M + A	PMS + M + A	PMS + C	M + C	PMS + M + C
—	0	++	+	0	++	+
Urea (3.25 M)	0	++(+)	+	0	++(+)	+
Fluoropyruvate (1 mM)	0	++(+)	0	0	++(+)	0
HgCl ₂ (1 mM)	0	0	0	0	0	0

^aWith PMS/amobarbital as well as with PMS/cyanide no detectable staining was registered.
+++ = heavy staining; ++ = moderate staining; + = low staining, 0 = no detectable staining.
PMS = phenazine methosulfate, A = amobarbital, M = menadione, C = cyanide.

- 1d. Basic medium + PMS + cyanide
- 1e. Basic medium + menadione + cyanide
- 1f. Basic medium + PMS + menadione + cyanide

Sections were incubated in each of the media with and without one of the following inhibitors: 1 mM fluoropyruvate (FP), to inhibit the aerobic type of LDH (16); 3.25 M urea, to inhibit the anaerobic type of LDH (10,15); and 1 mM HgCl₂, to inhibit all LDH activity (15) (Tables 1–3).

Incubation for NADH diaphorase (E.C.1.6.99.3) was performed according to Chayen et al. (4), with a medium (medium 2) having the following composition: 4 mM NBT and 5 mM NADH dissolved in 50 mM glycylglycine buffer (pH 8.0).

Incubation for LDH was done according to Barka and Anderson (2) in order to demonstrate diffusion artifacts due to NADH-diaphorase interference, which has been reported by Farber et al. (7) to occur in aqueous media. Sections were incubated in a medium (medium 3) consisting of 100 mM D,L-lactate with 7.5 mM NAD, 5 mM NBT, 5 mM MgCl₂, and 10 mM cyanide dissolved in 25 mM phosphate buffer (pH 7.2). In all incubation, sections were covered with the medium and incubated in a moistened chamber in the dark at 37°C for 10 min. The reaction was stopped by careful washing with ice-cold distilled water, and the sections were mounted in glycerine jelly. The sections were immediately analyzed for staining, and were photographed.

Controls. Serial sections were concomitantly incubated in media lacking substrate or coenzyme.

Table 2. Same conditions as in Table 1 with semiquantitative evaluation of LDH staining in spinosum cells of oral epithelium^a

Medium	1a	1b	1c	1d	1e	1f
Inhibitor	PMS + A	M + A	PMS + M + A	PMS + C	M + C	PMS + M + C
—	++	0	++	++(+)	(+)	++(+)
Urea (3.25 M)	0	0	(+)	0	0	0
Fluoropyruvate (1 mM)	(+)	0	0	+	(+)	+
HgCl ₂ (1 mM)	0	0	0	0	0	0

^aWith menadione/amobarbital no staining was detectable. Note also urea inhibition of spinosum staining.

Table 3. Same conditions as in Table 1 with semiquantitative evaluation of LDH staining in heart cells^a

Medium	1a	1b	1c	1d	1e	1f
Inhibitor	PMS + A	M + A	PMS + M + A	PMS + C	M + C	PMS + M + C
—	0	++(+)	0	++(+)	++	++(+)
Urea (3.25 M)	0	+	0	+++	++	+++
Fluoropyruvate (1 mM)	0	+	0	++	++(+)	++
HgCl ₂ (1 mM)	0	0	0	0	0	0

^aNote the difference in response to PMS/amobarbital and PMS/cyanide of heart cells as compared with basal cells in Table 1.

Chemicals. Polyvinyl alcohol (grade G04/104) was purchased from the Cytochemical Co., Edgeware, Middlesex, UK; cyanide from BDH Ltd.; amobarbital (Amytal) from SERVA, and glycylglycine and HgCl₂ from Merck. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. It was found imperative that only fresh supplies of PMS be used, and that they be stored in the dark below 0°C.

Results

General Observations

Mucosal epithelium, skeletal muscles, liver, kidney, brain, and tooth buds showed staining with most of the media used, although a great variation was observed in the staining pattern between sections incubated in the different media. The thickness of the sections did not permit an analysis of individual cells, and a detailed analysis of staining intensity was therefore restricted to the oral epithelial layers, which were compared with heart and skeletal muscles within the same section. Through analysis of the staining pattern in a whole-body section, it was possible to determine how LDH staining in the different organs was affected by the presence of each individual additive (see Fig. 2a, b). The staining results with the different media

Figure 1. Details of oral palatal mucosa from freeze-dried sections of 10-day-old rat stained for LDH in media 1a–f and in media 2 and 3. Asterisk indicates spinosum layer and arrowheads point at basal layer of epithelium. CT indicates connective tissue of lamina propria. Original magnification ×400. (a) Oral mucosa incubated with PMS/amobarbital (medium 1a). Note staining in spinosum/granulosum layers and lack of staining in basal layer. Connective tissue unstained. (b) Incubation with menadione amobarbital (medium 1b). Staining is mainly seen in the basal layer. A slight staining is also seen in the connective tissue. (c) Incubation with PMS cyanide (medium 1d). Similar staining as in a. No staining of connective tissue. (d) Incubation with menadione cyanide. Staining is similar to that seen in b. No staining of connective tissue. (e) Incubation with PMS menadione amobarbital (medium 1c). Similar staining pattern as in a and c, although with a lower intensity staining of the spinosum/granulosum layers and a very low-intensity staining of the basal cells. (f) Incubation with PMS menadione cyanide (medium 1f). Staining pattern similar to the one seen in e. (g) Incubation for LDH in medium 2. Intense staining is seen in the basal layer, while the spinosum/granulosum layers show only little staining (cf. b and d). (h) Incubation for NADH diaphorase in medium 3. Note intense staining in basal layer. The spinosum/granulosum layers show little staining (cf. g).

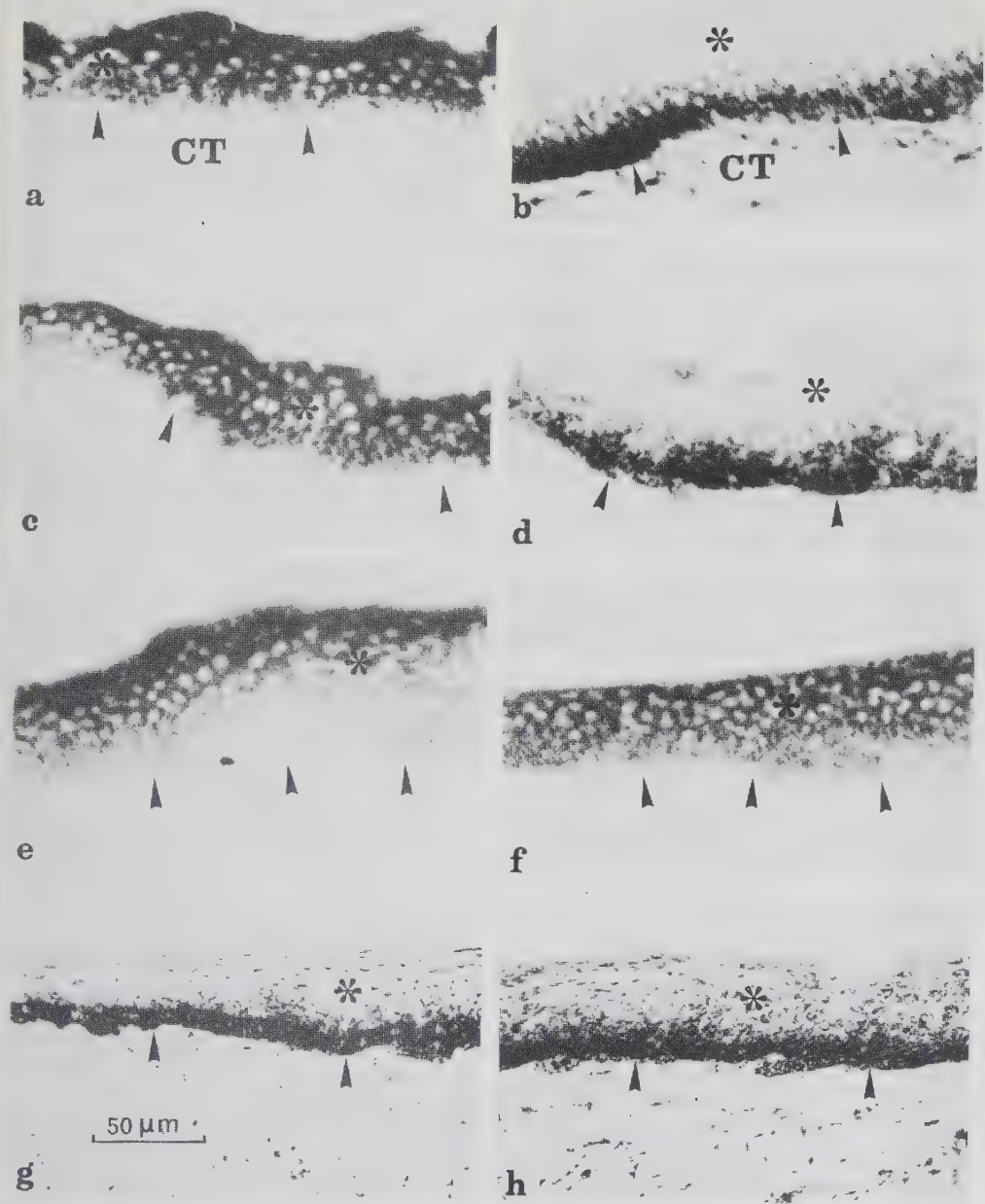


Figure 1

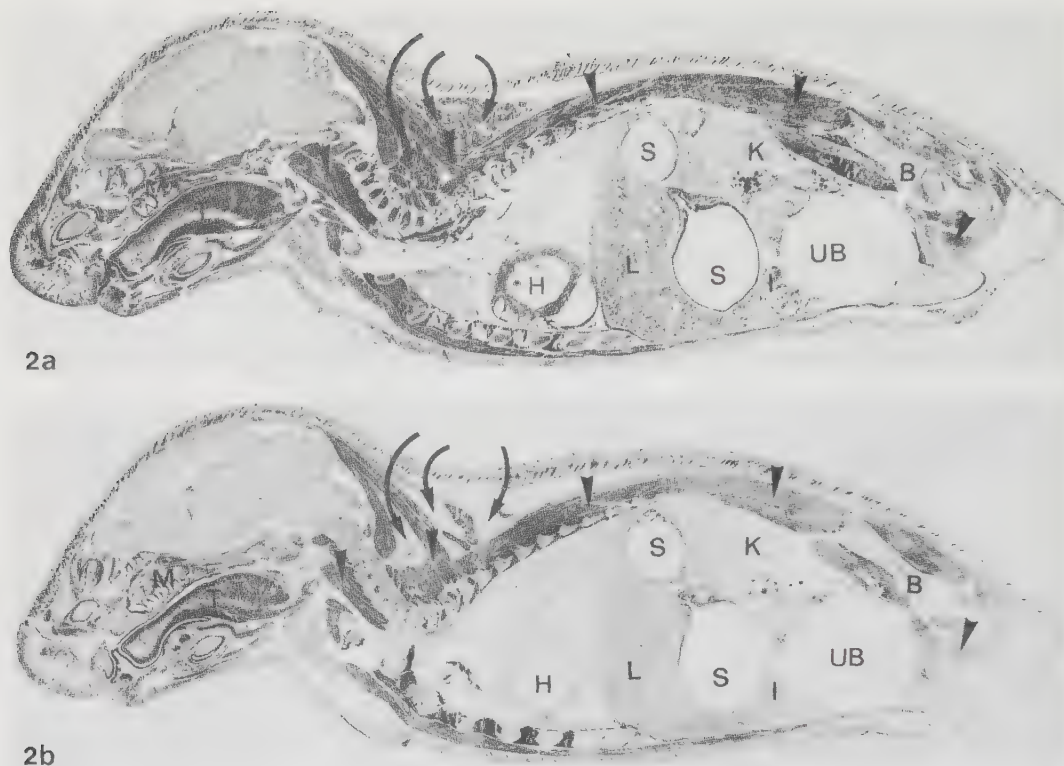


Figure 2. Serial 15- μ m thick sections of 10-day-old rat stained for LDH in different media. M = molars; T = tongue; H = heart; L = liver; K = kidney; S = stomach; I = intestines; UB = urine bladder; B = bone; arrows = brown-fat depots; arrowheads = skeletal muscles. Original magnification $\times 2.5$. (a) Section stained in LDH medium 1d with PMS and cyanide. There is a general but varied staining reaction in most tissues. The strongest staining reaction is seen in brown-fat depots, heart, and skeletal muscles. Also note the staining reaction in tooth-forming cells. (b) Section stained in medium 1a with PMS and amobarbital. The staining reaction is weaker as compared with that in a. No staining reaction is seen in brown-fat depots, heart, or kidney. A moderate staining reaction is seen in skeletal muscles and tooth-forming cells.

in oral epithelium, heart, and skeletal muscles are given in detail below, and for oral epithelium and heart also in Tables 1–3.

With medium 1a (PMS and amobarbital) an intense staining was found in the spinosum/granulosum layers of the oral epithelium, while the basal layer was almost unstained (Fig. 1a). A few skeletal-muscle cells exhibited an intense staining, while the majority of them stained weakly, and some were unstained. Heart tissue did not stain at all (Fig. 2b). With medium 1b (menadione and amobarbital), the basal layer of the oral epi-

thelium stained distinctly, while the spinosum/granulosum layers were mainly unstained (Fig. 1b). Nearly all skeletal-muscle cells, as well as heart-muscle cells, stained moderately and to an equal extent. Medium 1c (PMS, menadione and amobarbital), resulted in a staining pattern not unlike that seen with medium 1a. However, an additional slight staining of the basal layer in oral epithelium was noticed (Fig. 1e), while heart cells did not stain at all (Tables 1–3). With medium 1d (PMS and cyanide added to the basic medium) there was an intense staining of many tissues. Most conspicuous was the staining of heart cells. The basal layer of oral epithelium was unstained. The spinosum/granulosum layers were only moderately stained (Fig. 1c), as were many different cells, such as skeletal-muscle cells (Fig. 2a). The use of medium 1e, containing menadione with cyanide, seemed to result in a staining pattern similar to that seen with medium 1b (Fig. 1d). Medium 1f, containing both PMS and menadione with cyanide, seemed to counteract the stain-reducing effects of PMS in heart cells seen with media 1a and c. In oral epithelium, however, this was not the case and the basal cells were largely unstained (Fig. 1f).

The addition of inhibitors to the different media caused a considerable reduction of LHD staining generally. Mercuric chloride (HgCl_2) was the most powerful stain-reducing agent,

inhibiting all staining in all tissues. Fluoropyruvate did not affect all tissues, and only partly inhibited the LDH staining in the basal epithelial layer, the heart, and some of the skeletal-muscle cells. Urea inhibited the staining of the spinosum/granulosum layers and most skeletal-muscle cells. However, urea also seemed to enhance the LDH staining in the basal layer of the oral epithelium, the pulp tissue of the tooth buds, and the tubule cells of the kidney.

Sections incubated with medium 2 for NADH diaphorase and with medium 3 for LDH according to Barka and Anderson (2) showed an almost identical pattern with staining in the oral epithelium mainly involving the basal cells. Additionally, NADH-diaphorase staining was seen in some of the lower spinosum cells (Fig. 1g, h).

In the control sections, there was always a very weak staining when NAD^+ was omitted. However, when lactate was left out and amobarbital was replaced by cyanide, a considerable background staining was seen in many tissues, particularly the granulosum cells of keratinizing epithelium. This was considered to be caused by "nothing dehydrogenase" (26). It also occurred after a very short incubation time, and was difficult to avoid even with the medium modifications suggested by McMillan (15) and Jacobsen (10).

Discussion

Phenazine methosulfate and menadione are substances that have a redox propensity and are used in dehydrogenase histochemistry in order to mediate between the reduced coenzyme and NBT. Theoretically, this leads to enhanced precipitation in the tissues of formazan, an insoluble purple stain, at the site of the enzyme reaction (see the reaction scheme in Fig. 3). Since this also enhances the enzyme reaction rate, incorrect staining through diffusion is thus avoided (for a review of dehydrogenase histochemistry see refs. 12, 20). However, PMS improves LDH staining in tissue sections only if it is used under strictly anaerobic conditions; otherwise, it will inhibit LDH activity, as shown by McMillan (15). He recommended an alternative to the complicated anaerobic incubation technique by simply adding cyanide to the incubation medium, and also warned against the combination of PMS with amobarbital, which does not prevent the inhibitory effects of PMS. The results in the present study show that in tissue sections, PMS may have inhibited LDH staining in basal cells of the developing rat oral epithelium. However, in contrast to the findings of McMillan (15), the use of cyanide instead of amobarbital did not reverse the negative effects of PMS; and nor do anaerobic incubation conditions (22). In heart tissue, however, the inhibitory effect of PMS was reversed when amobarbital was replaced by cyanide, which is in accordance with previous results (15). Obviously, the LDH of heart tissues and the LDH of epithelial basal cells react differently to PMS.

Menadione, the second frequently used mediator in dehydrogenase histochemistry, particularly improves staining of the aerobic type of LDH in tissue sections (17). In this study, menadione, whether used with amobarbital or with cyanide, resulted in the staining of basal cells but not of spinosum/granulosum cells.

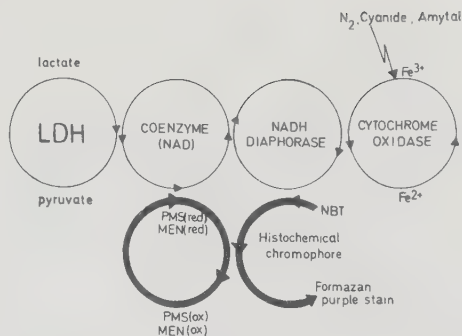


Figure 3. Reaction scheme for demonstrating LDH staining in tissue sections. PMS = phenazine methosulfate; MEN = menadione; NAD = nicotinamide adenine dinucleotide.

The staining reaction of basal cells observed in the present study corresponds well with that seen in previous studies of LDH staining in oral epithelium (9, 11, 13, 18), all of which were performed in aqueous media. However, the staining obtained for LDH after incubation in aqueous media may partly be caused by NADH-diaphorase activity (7). The similarities in the localization of LDH following incubation in a stabilized menadione-containing medium or in an aqueous medium (2), and in the occurrence of NADH diaphorase, are suggestive of an interference by NADH-diaphorase activity, and consequently complicate the interpretation of basal-cell LDH staining.

A further point is that when using menadione with PMS, as suggested by Meijer (17) and by Lojda et al. (12), one would expect basal-cell staining of at least the same intensity as is seen after the use of menadione alone, and of spinosum/granulosum-cell staining of at least the same intensity as is seen after the use of PMS alone. However, the spinosum/granulosum cells appeared less stained and the basal cells remained unstained with PMS/menadione, whether the latter was used with cyanide or with amobarbital. This may indicate an inability of menadione to improve LDH staining, and an inhibitory effect of PMS on rat oral epithelial LDH. Alternatively PMS and menadione may also influence NADH-diaphorase staining (see above).

On the basis of the present observations, there may be at least two different types of LDH in rat oral epithelium: one in the basal cells and sensitive to PMS, and the other in the spinosum/granulosum cells and undetectable with menadione. The basal-cell LDH staining was slightly affected by the addition of fluoropyruvate, which may indicate that these cells contain the aerobic type of LDH (17), while the spinosum/granulosum-cell LDH seemed unaffected by fluoropyruvate. Both basal-cell LDH and heart-cell LDH were inhibited by fluoropyruvate, yet the two appear to be different LDH isozymes, since inhibition of the heart-cell LDH by PMS could be prevented by the addition of cyanide, while inhibition of the basal-cell LDH could not (see above).

Urea inhibited the spinosum/granulosum-cell LDH, which

indicates that this is mainly an anaerobic LDH (15). The mechanism behind the urea enhancement of LDH staining (such as with basal- and heart-cell LDH), which was also observed in this study, is unknown.

Future findings may more satisfactorily explain the biologic significance of different LDH isozymes in the different layers of oral epithelium. However, the aerobic types of LDH in basal cells and the anaerobic type in spinosum cells may well reflect maturational changes in LDH production, possibly provoked by a shift in the intracellular NAD/NADH ratio, due to oxygen depletion (5). Maturational changes in LDH isozyme composition have previously been observed in other tissues of the rat (8), as well as in other species (3).

In conclusion, it seems that the difference in the localization of rat oral epithelial LDH observed in previous studies (9,11,13,18) versus those in the study of Sjögren et al. (22) were mainly due to differences in histochemical techniques, where neither method demonstrated all LDH isozymes. Discrepancies between the various histochemical methods in demonstrating LDH activity are obvious, and it seems that no single method is reliable for totally demonstrating the LDH in every kind of tissue. Also, the theoretical basis for the use of PMS, menadione, cyanide, and amobarbital in the incubation medium seems inadequate, and their influence on the incubation conditions is probably more complex than hitherto documented.

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LACTATE DEHYDROGENASE ISOZYMES IN DEVELOPING RAT ORAL MUCOSA. A COMPARATIVE STUDY OF LDH BIOCHEMISTRY AND HISTOCHEMISTRY.

By

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Running title: Lactate dehydrogenase isozymes in rat oral mucosa.

Key words: Cyanide, histochemistry, inhibitors, isozymes, lactate dehydrogenase, oral mucosa.

Abstract - Histochemical lactic acid dehydrogenase (LDH) staining methods seems to be inadequate in demonstrating the total LDH activity in tissue sections.

In this study analysis were made of LDH tissue staining methods applied on LDH zymograms. The menadione mediated LDH staining seen in zymograms shows that the menadione mediated LDH staining of tissue sections can hardly reflect true LDH activity. Also addition of cyanide slightly inhibited LDH activity. The cyanide inhibition was confirmed with LDH assay and found to be competitive in character. It is concluded that cyanide and menadione should be replaced by agents acceptable from both a histochemical and a biochemical point of view.

Based on the findings of this study the presence of LDH in oral epithelium was analyzed and evidently LDH of oral epithelium is basically anaerobic in character and found chiefly in spinosum/granulosum layers and only sparsely in the basal layer.

INTRODUCTION

Lactic acid dehydrogenase (LDH E.C.1.1.1.27.) is a tetramere molecule, with the monomere being of either A (former M) or B (former H) type, forming five different isozymes (28). In the rat the anaerobic A type is primarily found in skeletal muscles and liver cells, and the aerobic B type is found in heart and kidney cells (3). However, in most organs all five isozymes are present and form individual LDH 1-5 zymograms for each individual organ and species (3).

LDH isozymes have most commonly been studied by means of electrophoresis (29), but a more precise cellular localization is usually not possible to obtain with this technique and hence histochemical methods have been tried. McMillan (30) developed a technique, which made it possible to demonstrate different LDH isozymes in tissue sections by applying urea and pyruvate inhibition tests. However, it was later shown that this and other histochemical methods were insufficient in demonstrating all LDH isozymes in tissue sections (41). This may be due to endogenous, inhibiting factors of tissue sections, of which very little is known. It may also be due to incubation factors such as temperature variations, oxygen interference (30) or enzyme and coenzyme diffusion from the tissue section into the incubation solution (11,35). To control temperature and atmosphere problems, incubations have been recommended to be carried out in an anaerobic atmosphere and at a constant temperature of 37°C. In order to prevent leakage from the sections and diffusion, the usage of a viscous incubation medium has been recommended (1,30,37). There is also a possibility that the ingredients of the incubation medium may inhibit the enzyme reaction. Current histochemical media used for LDH demonstration have come to include a number of substances over the years. Such substances are phenazine

methosulphate, menadione, amobarbital and cyanide, added to improve the staining reaction (5,6,13,25,30,31,32,44). The purpose of this study was to analyze some of the effects phenazine methosulphate, menadione, amobarbital and cyanide have upon the activity of LDH in oral mucosa. For reference purposes specific organs such as heart, liver and skeletal muscles with a well known isozyme pattern were also included in the study.

MATERIALS AND METHODS

TISSUE PREPARATION

10-day-old rats were killed by decapitation and their buccal mucosa, liver, heart and skeletal muscle (hind leg) removed. The buccal mucosa was freed from underlying tissues by dissection with a blunt instrument and excised all in order to minimize muscle tissue contamination. The tissues were cut to smaller pieces by a pair of scissors and washed in saline, mixed with 9 volumes of 0.01 M Tris-HCl, pH 8.0 + 1 mM B-mercapto ethanol + 1 mM dithio-treitol and homogenized. Homogenization was done in a Polytrone homogenizer at half speed for 15 seconds in an icebath, after which samples of the homogenates were collected. The remainder was centrifuged (40.000 g) for 1 hour in a Sorval Superspeed. The supernatants were collected and together with samples of the homogenates stored at -85°C until further use.

ELECTROPHORETIC SEPARATION OF LDH ISOZYMES

10 μl of supernatant from the different tissues were layered on top of 5.5% acrylamide gel rods (8,9). Additional 5.5% acrylamide gel was carefully layered on top of the extract in some rods to permit detection of cathodal migration of LDH. The electrophoresis was conducted in a Pharmacia vertical electrophoresis apparatus, with 0.05 M tris- and 0.38 M glycine, pH 8.3, as electrode buffer for 45 minutes and 2.5 mA/gel rod.

HISTOCHEMICAL INCUBATION OF GEL RODS

After electrophoresis the rods were removed from the glass tubes and incubated in a medium containing 100 mM sodium lactate, 1.5 mM nicotinamide adenine dinucleotide (NAD), 0.5 mM MgCl_2 , 0.3 mM nitroblue tetrazolium (NBT), and 0.08 mM phenazine methosulphate (PMS) dissolved in 0.125 M phosphate buffer, pH 7.4, for 45 minutes at $+37^\circ\text{C}$ (9). To this basic medium was added either 10 mM amobarbital or 1 mM cyanide. Incubations were also done, where PMS was replaced by 1.2 mM menadione (0.33 mg/ml) in the medium (31,32).

Some gel rods were incubated for NADH diaphorase (E.C. 1.6.99.3) in a medium of 1.2 mM NBT, 1 mM sodium cyanide, 10 mM MgCl_2 and 2.8 mM NADH dissolved in 100 mM phosphate buffer pH 7.4 (25).

Control incubations were done by omitting either substrate or coenzyme.

GEL SCANNING ANALYSIS

After incubations the gel rods were washed in ice cold distilled water, fixed in 7.5% acetic acid, photographed and analyzed at 557 nm in a Beckman 24 spectrophotometer with an adapted recording gelscan. The length of the LDH isozyme peaks were measured and used to calculate the relative content of the individual isozyme bands 1-5. Also the relative content of LDH A and B was calculated, based on the assumption that the content of subunit A in LDH 5 was 100%, in LDH 4 75%, in LDH 3 50% and in LDH 2 25%. Theoretical estimation of a possible influence from buccal muscle LDH contamination on mucosal LDH zymogram staining was based on the assumption, that muscular LDH 1-5 contaminated proportionally equal its counterpart LDH 1-5 in the mucosal zymogram.

LDH ASSAY OF TISSUE EXTRACTS

Tissue homogenates and supernatants were assayed for LDH in 50 mM tris-HCl, pH 8.9, and 1 mM NAD in a final volume of 3 ml. Before incubation, 20 μ l tissue extract was added to the preheated incubation medium (37°C). The enzyme reaction was started by adding 200 mM lactate to the incubation medium (2,4,27) and monitored at 340 nm in a recording Hitachi 100-60 spectrophotometer equipped with a thermostatically controlled cell compartment and automatic sample changer. Thus, it was possible to simultaneously follow the enzyme reaction, the control reaction and also the influence of added cyanide or amobarbital. The influence of 0.5 and 1 mM cyanide was studied at varying concentrations of lactate (3.5 and 10 mM) in a 50 mM tris-HCl buffer, pH 8.9, with 1 mM NAD and 1 international unit of rabbit LDH M_4 ($=A_4$)/ml added to the medium. Control reactions of the assay was monitored simultaneously (see above and Figures 2a-d), either without substrate or without amobarbital and cyanide or both. Control reactions without extract and substrate were also done.

HISTOCHEMISTRY OF TISSUE SECTIONS

Incubations for the demonstration of LDH and NADH diaphorase activity of 10-day-old rats were done on whole body, 20 μ m thick, freeze-dried tissue sections obtained through a technique of Ullberg (42). The sections were incubated for 15 minutes at 37°C, in 0.05 M glycylglycine buffer, pH 8.0, with 22% polyvinyl alcohol (PVA), 50 mM sodium DL-lactate, 5 mM NBT, 2.5 mM NAD and 0.65 mM PMS in the incubation medium (1,6). To this was added 1 mM cyanide or 10 mM amobarbital. Sections were also incubated in a medium, where PMS was replaced by menadione (1.2 mM) (31,32).

Histochemical demonstration of LDH activity under anaerobic incubation conditions was also done. The incubation

medium was prepared by dissolving 22% PVA in 0.05 M glycyl-glycine buffer, pH 8.0. The solution was exhaustively degassed and saturated with nitrogen, before lactate, NAD, NBT and PMS of the same concentrations as above, were added. No amobarbital or cyanide was added. Just before the medium was poured over the sections, nitrogen was bubbled through once more. The sections with medium were placed in a two valve vacuum chamber, that had been heated to 37°C. The chamber-air was evacuated immediately and replaced with nitrogen gas; a procedure which was repeated four times to optimize anaerobic conditions. The chamber was placed dark at 37°C for 40 minutes, after which the incubation was ended and the sections carefully washed with distilled water, mounted in glycerine jelly, analyzed for staining and photographed.

NADH diaphorase activity was demonstrated by incubating the sections in 0.1 M phosphate buffer, pH 7.4, with 1.2 mM NBT, 1 mM sodium cyanide, 10 mM/MgCl₂ and 2.8 mM NADH (25).

Control incubations were included regularly in the staining procedures by omitting either the substrate or the coenzyme.

CHEMICALS

Cyanide was purchased from BDH Ltd U.K., polyvinylalcohol grade G04/140 from Cytochemical Co. U.K. and amobarbital (Amytal^R) from Serva GmbH W.G. All other chemicals, including rabbit muscle LDH M₄, were obtained from Sigma Chemical Co., Missouri, U.S.A.

RESULTS

ELECTROPHORESIS

All zymograms of the various tissue extracts, except liver, contained five different LDH bands when stained in

the basic LDH medium. In general, addition of amobarbital resulted in strong staining, while addition of cyanide led to less staining compared with the staining obtained with the basic medium (Figures 1a-d).

When staining was done for LDH with menadione containing medium or for NADH diaphorase, a completely different set of staining patterns appeared with only two bands detectable in the different tissue gel rods (Figures 1a-d). Menadione inhibited three of the five LDH isozymes. In addition, increased background staining of the gels was also seen with menadione containing medium.

GEL SCANNING ANALYSIS

Gel scanning analysis showed that the relative amount of anaerobic LDH varied between the different tissues. Oral mucosa and skeletal muscle extract contained roughly similar proportions of anaerobic LDH (65%), while liver extracts had the largest proportion of anaerobic LDH (90%) and heart extract the lowest (45%).

Control incubations of gel rods without the substrate resulted in a weak staining in the region of each different LDH 1-5 staining band and was subtracted from the peaks in the gel-scan analysis.

LDH ASSAY OF TISSUE EXTRACTS

All assays were done three independent times and gave allways the same result. No statistics were done.

Homogenates and supernatants from oral mucosa, skeletal muscles, liver and heart contained varying amounts of LDH. Only data of oral mucosa is given in figures 2a-d. There were little differences between LDH activity in homogenates and in supernatants within the individual tissue. However, there was a slow increase of absorbance at 340 nm with cyanide present in the reaction medium that was not contributable to LDH activity (Figures 2a-b).

With constant amount of cyanide and NAD and no lactate

present, but with varying amounts of tissue extract, no differences in the increase of absorbance at 340 nm was seen (Figure 3a). When the concentration of cyanide was varied and incubated with 1 mM NAD and homogenate or supernatant in distilled water, the rate of increased absorbance varied with the increasing amount of cyanide (Figures 3b and c). Similar studies were done, where cyanide was replaced by amobarbital and no detectable non-LDH mediated increase in absorbance at 340 nm was found (Figures 2c and d). Apart from that, the results were much the same as with cyanide.

Inhibition studies with LDH M_4 (A_4) revealed that the LDH activity was competitively inhibited by cyanide (Figure 4). The Michaeli-Menten constant for the commercial LDH M_4 was estimated from the Lineweaver Burke plot to be 10 mM lactate (Figure 4). Assays of extracts without the substrate and cyanide or without the coenzyme and cyanide showed no increased absorption at 340 nm (Figures 2a-d).

HISTOCHEMISTRY OF TISSUE SECTIONS

Sections incubated for LDH in the presence of cyanide showed a more intense staining, than sections incubated with amobarbital in the medium. With amobarbital no staining was found in kidney or in heart and a marked decrease in staining was found in the liver. With amobarbital and cyanide in the medium, LDH staining was seen primarily in the spinosum/granulosum layers of the oral mucosa. The basal layer was stained very lightly. Sections stained for LDH with menadione containing medium and for NADH diaphorase showed similar staining patterns of the oral mucosa (Figures 5b and c), with intense staining of the basal layer and decreased staining towards the upper layers.

Sections incubated under anaerobic conditions showed LDH staining in the spinosum and granulosum layers of the

oral epithelium, with no staining of basal layer (Figure 5a). The intensity of the staining varied slightly from region to region of the oral cavity, but variations were seen also within limited parts of individual regions. The variations occurred seemingly at random without relation to any specific structure.

In sections incubated in control medium without lactate, only a weak background staining was seen, which was subtracted from the staining in the analysis. The background staining was increased however when cyanide was present in the medium. Background staining was considered to represent "nothing dehydrogenase staining" described by Zimmerman and Pearse (39).

DISCUSSION

The different LDH zymograms of oral mucosa and other tissues found with the basic LDH staining are in good agreement with those of previous studies (3,14,43). However, the addition of PMS, menadione, amobarbital and cyanide affected the LDH zymogram staining of gels in a different way, compared with what has been reported of their effects on LDH staining in tissue sections (5,17,30,31,32,41).

Thus, zymograms stained for LDH activity with the menadione containing medium, seemed to differ very little from those stained for NADH diaphorase activity. Either the menadione inhibited all the LDH isozymes and the staining reaction was due to the NADH diaphorase, or the menadione selectively inhibited the LDH isozymes 1,2 and 5. Be that as it may, as the present results suggest, menadione should not be used in histochemical LDH staining media.

Incubation in amobarbital containing media selectively inhibited LDH activity of heart and kidney in tissue

sections, enhanced the activity in homogenates including that of heart, while activity in supernatants was more or less unaffected. The inhibiting effect of amobarbital seems thus to be connected with specific factors of the intact heart tissue rather than with the catalytic activity of the heart LDH. In this context interesting observations have been made on the binding behaviour of the LDH molecule. LDH was shown in vitro to be attached to plasma membranes in an inactive state from which it could be released by alterations of substrate and co-enzyme concentrations (16,34). It was also released and activated by tissue homogenization alone (10). Although the exact mechanism behind the binding and release of the LDH in vivo is not clear, the previous results (10,16,34) and the present implies that the amobarbital inhibition of the LDH in intact tissue may have acted through interference with the release of the LDH molecule from the plasma membranes. The differences in effect of amobarbital on the LDH activity found in homogenate and supernatant may have been caused by interfering mitochondrial activity present in the homogenates and blocked by amobarbital. In supernatants this effect could not occur. Hence amobarbital may be used in LDH assays and in LDH zymogram histochemistry but not in LDH histochemistry of tissue sections as previously shown (30).

With cyanide the result was different. Cyanide added to the LDH incubation medium enhanced the staining in tissue sections, reduced the staining of all LDH bands in zymograms and partly inhibited the enzyme activity of whole homogenates, as well as supernatants. This implies, that there are factors, other than the LDH, in the intact tissue, that are involved in the cyanide promoting effect of LDH staining. In addition, cyanide and NAD form complexes (7), which lead to an increased absorbance at 340 nm (7,46). In the LDH assay, this mimicks NADH formation, which has an absorbance maximum at 340 nm. The

NAD-cyanide reaction occurs independently of LDH (12,46). When cyanide binds to NAD it occupies the active site of the NAD molecule (12), which may have caused the competitive inhibition of LDH demonstrated in the present study.

Cyanide may also react with other components of the reaction medium and produce non-enzymatic staining. Thus it has been shown that cyanide is able to oxidize NADH in the presence of glyceraldehyde-3-phosphate and LDH (38), a reaction, which antagonizes the conversion of NBT to formazan. Cyanide can also spontaneously reduce NBT to formazan (20,39).

Considering the results of previous studies (7,12,20,38,39,46) and of the present, it seems, that addition of cyanide to a complex incubation medium must lead to a staining result extremely difficult to evaluate for LDH activity. On a practical level it may mean, that the cyanide concentration has to be reduced drastically from the conventional level of 1-25 mM (5,17,25,30,36). Alternatively, cyanide should be replaced by other agents acceptable from both a biochemical and a histochemical point of view.

From the results of this study also follows that the demonstration of LDH activity in basal cells of oral mucosa based on menadione staining must be reconsidered, since it may reflect NADH diaphorase staining. When preventing diffusion of LDH and NAD from the sections with PVA and applying anaerobic incubation conditions for the LDH reaction no indication of LDH activity was found in basal cells of oral epithelium. These findings are contrary to previous reports of LDH activity in oral mucosa, where the highest activity was found in basal cells (15,21,25,26,33). It seems that the major LDH activity in oral epithelium is localized at the spinosum/granulosum cells and is of anaerobic type A. This

means that in average oral epithelium have an energy metabolism that is anaerobic in character, a finding in accordance with previous studies of squamous epithelium metabolism (18,19,22,23,24,40) but that the basal cells are an exception of this, with an essentially aerobic metabolism (18,19,40) of which LDH takes only a small part, if at all.

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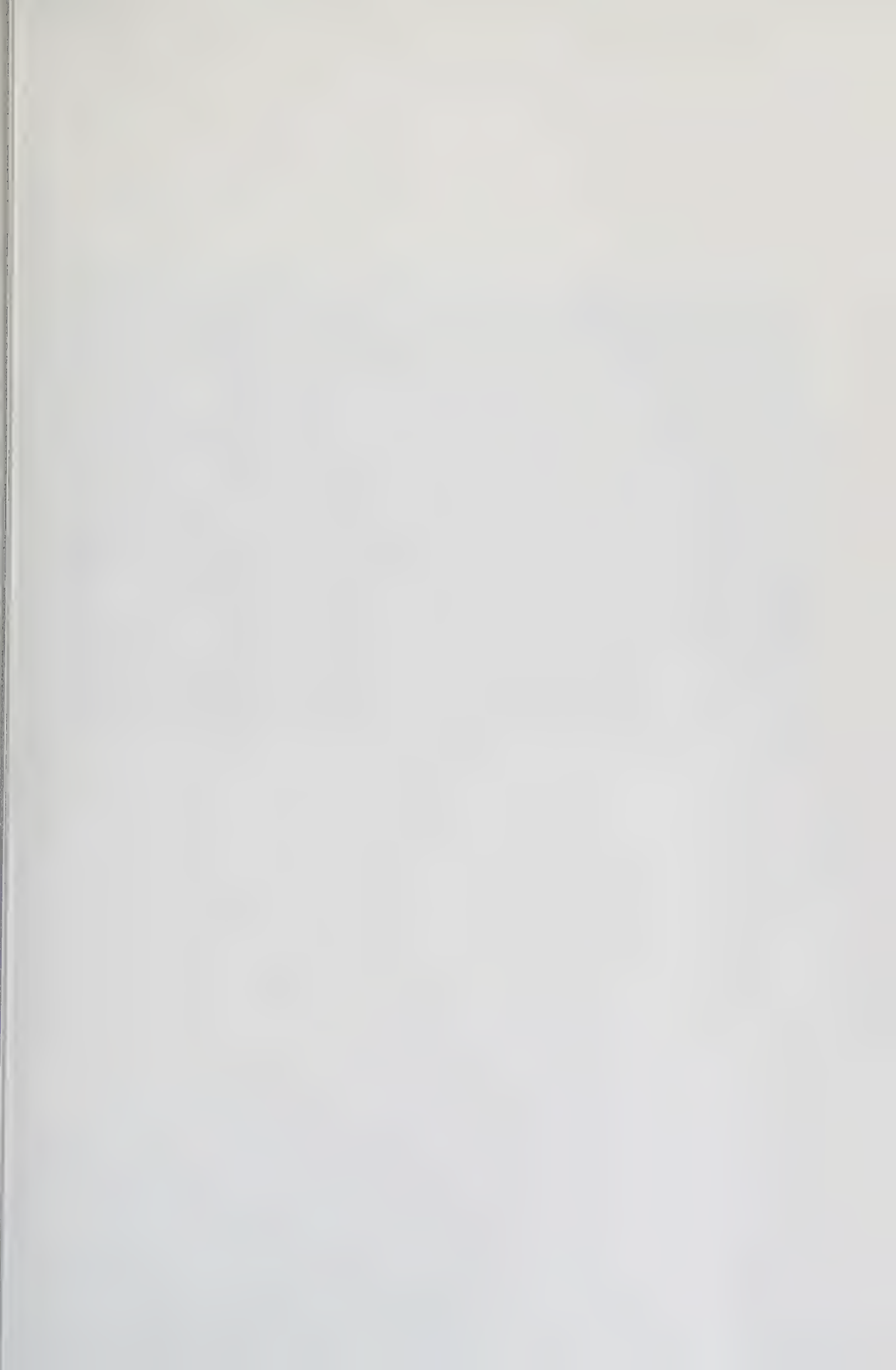


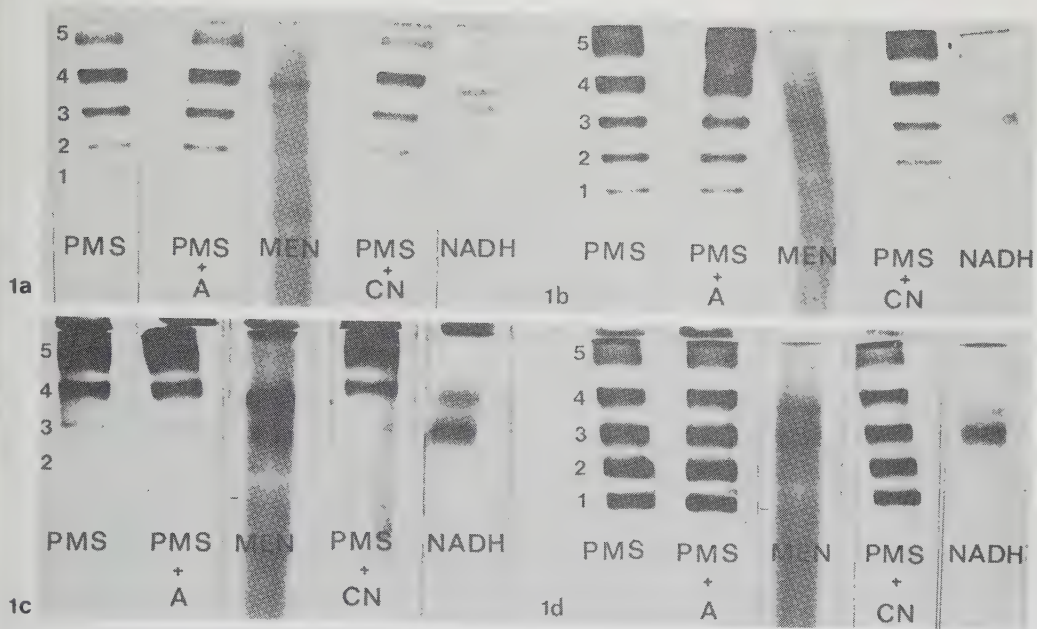
Figure 1a. Zymograms of mucosal extract obtained with PAGE and stained from left for LDH with phenazine methosulphate (PMS), PMS and amobarbital (A), menadione (MEN), PMS and cyanide (CN) in the incubation medium. The zymogram to the right is stained for NADH diaphorase (for details about the individual incubation medium see material and method).

Figure 1b. Zymograms of skeletal muscle extract. Staining is same as in fig. 1a.

Figure 1c. Zymogram of liver extract. Staining is same as in fig. 1a.

Figure 1d. Zymograms of heart extract. Staining is same as in fig. 1a.

1 = LDH 1, 2 = LDH 2, 3 = LDH 3, 4 = LDH 4, 5 = LDH 5.



Figures 2a-d. LDH assays of buccal mucosa extract from 10-day-old rats showing the differences between homogenate and supernatant and the effect of cyanide and amobarbital addition (assay conditions are as in material and methods).

2a. Twenty μ l of buccal homogenate incubated with: (1) Lactate + NAD + 1 mM cyanide + buffer. (2) Same as (1) without cyanide. (3) Same as (1) without lactate. (4) Same as (1) without both lactate and cyanide. Cyanide addition increases the absorption.

2b. Same conditions as in 2a with the exception of the homogenate, which was exchanged for the supernatant of buccal mucosa. No real difference from 2a is seen.

2c. Same as 2a, but with amobarbital instead of cyanide in the assay. Note that amobarbital addition stimulates the LDH reaction. Without the lactate, amobarbital does not seem to have any effect.

2d. Same as in 2b, but with amobarbital instead of cyanide in the assays. Note that amobarbital addition does not seem to have any effect on the LDH reaction when supernatant is used and compare with fig. 2c where homogenate is used in the assay.

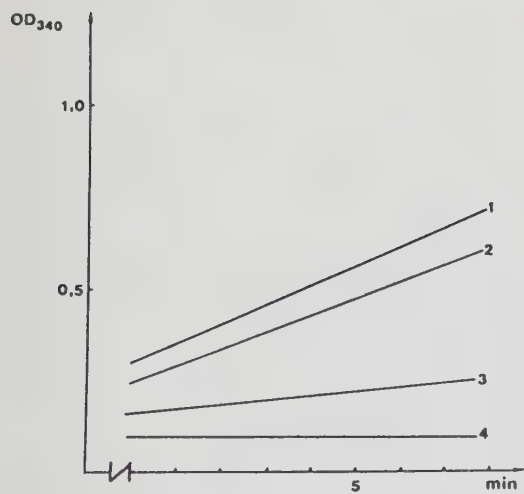


Fig 2a

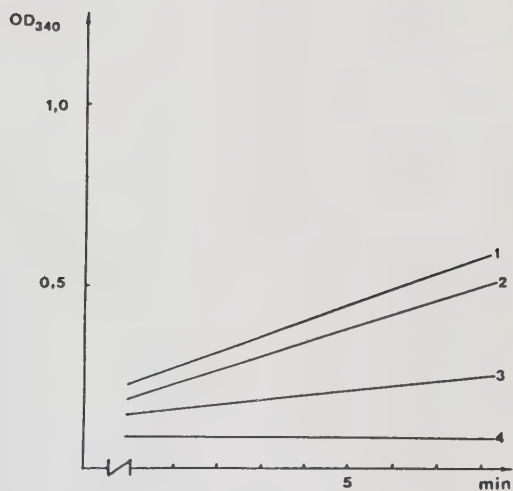


Fig 2b

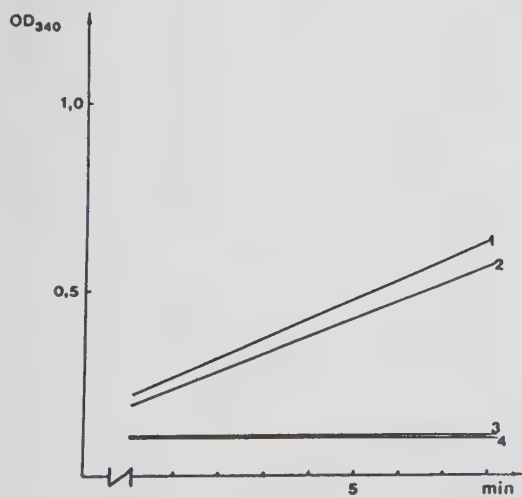


Fig 2c

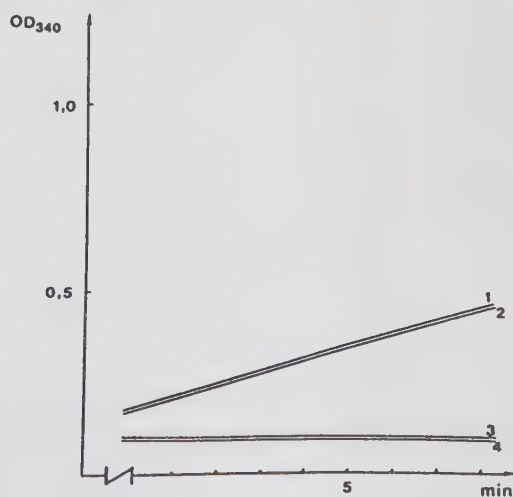


Fig 2d

Figure 3a. LDH assay (for details see material and methods) with 100 μ l (1), 50 μ l (2), 20 μ l (3) and 10 μ l (4) of oral mucosal supernatant in the presence of constant amount of cyanide (1 mM). No difference was observed with increasing amount of tissue extract.

Figure 3b. LDH assay with 20 μ l homogenate of rat oral mucosa in the presence of 10 mM (1), 5 mM (2), 2.5 mM (3) and 1 mM (4) cyanide. Note the increased absorbance at 340 nm with increasing amounts of cyanide.

Figure 3c. Same as 3b, but with 20 μ l supernatant of oral mucosa instead of homogenate. No difference between homogenate (3b) and supernatant (3c) was found.

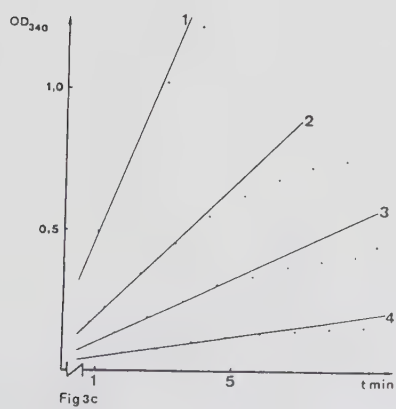
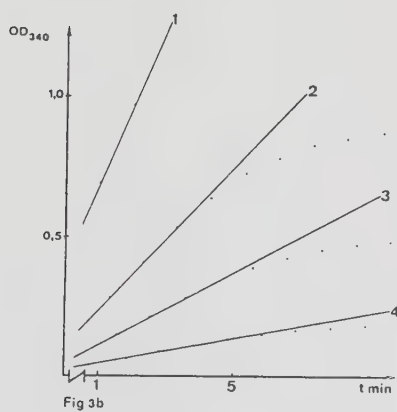
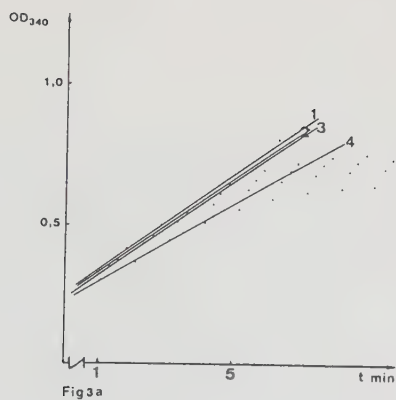


Figure 4. Lineweaver-Burke plot of LDH assay with 3 international units of commercial LDH type M_4 (A_4) incubated in 50 mM tris-HCl, pH 8.9 and with varying amounts of lactate (3, 5 and 10 mM) and cyanide (0 mM, 0.5 mM and 1.0 mM).

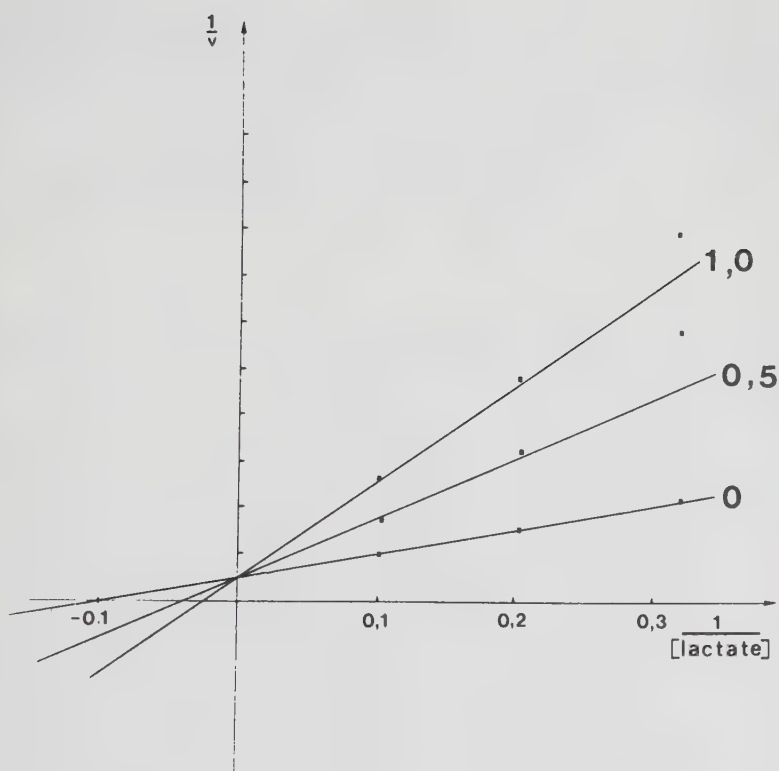


Figure 5a. Oral mucosa of 10-day-old rat incubated for LDH activity under strictly anaerobic conditions. Note the increase of staining towards the surface. No staining is seen in the basal layer.

Figure 5b. Same as 5a incubated for LDH activity with menadione and amobarbital containing medium (27). Note staining in basal and lower spinosum layers and in granulosum/corneum layers. The latter staining was considered to be due mostly to "nothing dehydrogenase" (39). No staining is seen in the upper spinosum layers.

Figure 5c. Same as 5a incubated for NADH diaphorase (20). Note staining in basal and lower spinosum layers. Weak staining is also seen in the upper epithelium layers.

ct = connective tissue of lamina propria, arrow heads = basal layer, asterisc = spinosum layer, g = granulosum layer, c = corneum layer. Bar = 25 um. X400

